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Docket No.: 1010/16959-US4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

HOWARD L. **WEINER** *et al.*

Serial No.: 08/469,492 Art Unit: 1645

Filed: June 6, 1995 Examiner: P. Duffy

For: **BYSTANDER SUPPRESSION OF AUTOIMMUNE DISEASES**

DECLARATION OF MATTHIAS G. VON HERRATH

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

Matthias G. Von Herrath declares and states as follows:

Considered 9/29/01 JMS

BACKGROUND INFORMATION

1. I hold both a Medical Doctor and a Doctor of Philosophy degree, conferred by Freiburg Medical School in 1988. Currently, I am employed as Associate Professor

by The Scripps Research Institute and I have held this position since 1999. Previously, I was Assistant Professor at the same institution from 1996 to 1999. For the past ten years, I have conducted extensive research in the immunology and immunomodulation of autoimmune diseases and particularly insulin dependent diabetes mellitus (IDDM).

2. I am an author of over 30 publications on the immunology of autoimmune diseases including several articles on bystander suppression. Further details of my qualifications are provided in the attached copy of my curriculum vitae (Attachment 1).

3. I submit this declaration in support of the patentability of the application identified above. I am not a co-inventor of this application and I hold no financial interest in either the Brigham & Women's Hospital or AutoImmune, Inc. which I understand have certain rights in the invention.

4. I am familiar with this application, including its specification, currently pending claims (Attachment 2), Office Action of March 29, 2000 and references cited therein, namely Tobin US patent 5,475,086; Herbert et al The Dictionary of Immunology, Fourth Edition, Academic Press 1995, pp17 and 88; Mueller, D.L. The Journal of NIH Research, 6:47-51, 1994; Hafler et al J. Immunol. 139:68, 1988; Cohen, I. et al AutoImmune Disease Models, p.2; and Paul et al Eds

"Fundamental Immunology" p.548.

5. Briefly, the claimed invention involves administration of a bystander antigen to suppress an autoimmune response associated with the autoimmune disease, i.e., to induce tolerance. The claims further specify that the bystander antigen is an antigen to which T-cells that modulate an abnormal autoimmune response are NOT sensitized, and that the mode of its administration is by mouth or nasal route.

QUESTIONS CONSIDERED

6. I understand that the United States Patent & Trademark Office has rejected the claims as non compliant with certain statutory requirements. Specifically, I understand that the Examiner is of the opinion that the patent application does not describe the invention as claimed in sufficient detail to enable a person of ordinary skill in the field of the invention to use it for all autoimmune diseases and bystander antigens encompassed by the claims.

7. I have been retained by patent counsel to consider the following questions objectively from the point of view of a person of ordinary skill in the field at the time this application was first filed, which I am told was February 28, 1992. These questions are:

QUESTION I: Does the specification provide sufficient support for the claims by circumscribing the area of application of the claimed invention and providing a representative number of working embodiments? Or does the fact that many of the antigens disclosed in the specification (and employed in experiments described therein) can be autoantigens detract from the generality of the statements in the specification such that a person of ordinary skill in the field would consider the invention to have been demonstrated only for glucagon and insulitis and for no other pure bystander antigen or autoimmune disease?

QUESTION II: On February 28, 1992, would a person of ordinary skill in the field reasonably expect the claimed invention to be operative in humans afflicted with various autoimmune diseases based on the animal model data presented in the specification? Based on the teachings of the specification and the general knowledge in the field at the time (2/28/92), would such a person know which bystander antigen to administer, when to begin, how much to administer, how often and for how long, or would such a person have to conduct an inordinate amount of experimentation--beyond what is considered routine experimentation in this field--in order to obtain this information?

QUESTION III: Are the admonitions in Mueller et al, *supra*, at p.49-50 regarding anergy induction applicable to the method of the present claims, i.e., bystander suppression?

<u>Question I</u>

8. I disagree with the Examiner on this question. The specification, through the experiments it describes, makes clear to a person of ordinary skill in the field that bystander suppression does not require administration of an antigen to which activated T-cells of the host are sensitized, but results in suppression of the

autoimmune (or autoimmune-like) response even when the administered antigen is a "pure bystander." A "pure bystander" is an antigen that is expressed in an organ or tissue that is the target of an abnormal autoimmune (or autoimmune-like) response but is not itself a target of the abnormal autoimmune response. The specification provides data with the following "pure bystander" antigens:

- glucagon orally administered to NOD mice (p48, lines 10-27 and p.49, Table 5)

- MBP orally administered to mice immunized with PLP (Example 6, p.49 lines 22-34, p.50, lines 1-13)[1]

- Ovalbumin orally administered to mice immunized with MBP (Example 2, pp36-45, especially p.42, line 6 through p.44 line 35)[2]

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[1]Even though MBP can induce EAE, in this experiment, PLP only was used for EAE induction. Moreover, the mice used for the experiment had to be healthy and would not have activated T-cells that would recognize MBP. Hence, MBP in this experiment fits the definition of bystander antigen that appears in claim 37.

[2]Ovalbumin ("OVA")cannot induce EAE. It is thus clearly a bystander antigen within the definition of claim 37. Moreover, ovalbumin is not expressed in the organ or tissue afflicted in EAE (which is the central nervous system) and is thus totally removed from the locus of autoimmune response. It follows that if OVA can induce bystander suppression, such suppression is a general immune phenomenon

- Noninducing fragments of MBP orally administered to mice challenged with MBP (Example 3, pp 45-46 and p.29, lines 5-10).

- Peptides derived from insulin which are not recognized by T-cells of NOD mice (p.48, lines 24-26 and p.49, Table 5).

9. The specification thus details experiments with at least five different "pure bystander" antigens (i.e. antigens not targeted by the immune response to be suppressed) administered in two different disease models. In particular, the use of OVA to suppress MBP-induced EAE is an important indication of the general applicability of the concept of bystander suppression because under normal circumstances (and unlike MBP and PLP) OVA is neither specific to the organ targeted by the aggressive autoimmune response, nor itself the target of an autoimmune or autoimmune-like response. In the OVA experiment detailed in the specification, Weiner et al engineered the OVA to be concentrated at the locus of the induced autoimmune-like response by injecting OVA into the mice at the site of their immunization with MBP, and thus created an artificial antigen "expressed" in

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independent of the context of a particular autoimmune response or autoimmune disease or animal model of such disease, as I discuss below.

the "target organ" where the abnormal immune response occurred. The inventors did this to show that regulatory T cells (which were elicited in the mice by feeding them OVA) "home-in" to the site of inflammatory immune response (where the OVA, which the regulatory cells recognize was artificially introduced) and reduce this response. Unlike MBP or PLP, OVA is not associated with any autoimmune disease since immunization with OVA does not cause disease; unlike glucagon, OVA is not even specific to an organ targeted by an abnormal autoimmune response. Thus, even though Weiner et al did not test their method on *every* autoimmune disease, in my opinion, a person of ordinary skill in the field would consider applicability of their method in any autoimmune disease to be likely in view of the experiments they did conduct and especially the OVA experiment: if immune regulation results by feeding an animal an antigen unrelated to any abnormal autoimmunity, and by mimicking a tissue-specific antigen through injection of this antigen to a site where autoimmune (or autoimmune-like) response is taking place, it follows that the immunoregulatory phenomenon observed by Weiner et al using bystander antigens is very likely a general one.

10. Even without taking into account these implications of the OVA experiment, the inventors have shown bystander suppression for four different antigens in two different autoimmune disease models (EAE and NOD diabetes), which in my view

supports the proposition that bystander suppression would abate autoimmunity in autoimmune diseases in general. Most important, the inventors have shown the regulatory immune response following ingestion of the bystander antigen to be associated with expression by regulatory T cells of nonspecific immunoregulatory factors, such as TGF-β. These factors are associated with the regulatory nature of the response and not with the nature of the antigen which the regulatory T cells recognize. This finding lends additional support to the generality of bystander suppression as elucidated by Weiner et al.

11. Thus, as a result of the experimental evidence contained in the specification, in February 1992, a person of ordinary skill in the field would have expected "pure" bystander suppression by oral or nasal route to be an effective means of suppressing autoimmune response regardless of the identity of the bystander antigen and regardless of the particular experimental system used. The experiments in the specification indicated that bystander suppression is a general immune phenomenon, even apart from the context of autoimmunity (see for example the experiment with OVA). When these experiments were presented to the scientific community, they were at first surprising but they were quickly recognized and have since become generally accepted as having elucidated an important and general immunoregulatory mechanism.

12. The experiments in the specification would have indicated to a person of ordinary skill in the field that bystander suppression using a "pure" bystander antigen is T-cell mediated suppression and, more likely than not, is effected in a manner similar to oral (and more generally mucosal) tolerance using autoantigens. In both forms of tolerance induction, ingestion of the antigen is accompanied by a regulatory rather than an aggressive immune response following antigenic stimulation. Hence, the person of ordinary skill would have expected bystander suppression to be effective when the bystander antigen is administered through the nasal mucosa, as the specification indicates at p.24, lines 25-29, similar to when an autoantigen is fed or nasally administered.

13. Thus, in my opinion, in 1992, the person of ordinary skill would have believed the statements in the specification and claims that "pure" bystander antigens by oral or nasal route would be effective to suppress an autoimmune response in humans.

Question II

14. Given the data in the specification, it is my opinion that a person of ordinary skill would clearly expect that oral or nasal administration of a "pure" bystander antigen (i.e., an antigen not recognized by activated auto-aggressive T cells of the patient) would suppress an abnormal autoimmune response in humans. The skilled

person would know from the specification that such bystander antigens can be selected from among peptide fragments of autoantigens which are not recognized by activated human T-cell clones. In my opinion, the assay for this purpose was feasible in 1992. (See, for example Hafler et al, J. Immunol. 139:68-72, 1987.) The amount of experimentation required would not be considered excessive because, in immunology, this type of experimentation was and is routine.

No guidance in spec for such.

15. The specification provides ample guidance as to how much bystander antigen to use as a starting point. For example, at p.25, lines 3-6, the specification provides a general range of 0.1mg-15mg/kg daily for nasal administration; at pp.26-27, the specification allows for developmental work which in 1992 could have been used to refine the dosages to be given to humans. The specification also provides detailed guidance in the form of dosages for PLP-induced disease at p.17, and even allows for differences between species of hosts. The fact that the various antigens may be autoantigens in certain individuals or experimental contexts would not alter the value of this disclosure. In other words, the effective dosage range of a bystander antigen which can also be an autoantigen can be used as guidance for the effective dosage range of a "pure" bystander. The principle behind use of each is the same: an antigen is administered mucosally in order to regulate immune response. The immunoregulatory effect in both instances is due to

elicitation of regulatory cells, and expression of regulatory factors, such as TGF-β. All of this was known with respect to autoantigens administered orally or nasally to achieve tolerance, as evidenced for example from the attached references: *Zhang, Z. J., et al*, ("Suppression of diabetes in Nonobese Diabetic Mice by Oral Administration of Porcine Insulin," *P.N.A.S. (USA), 88*:10252-10256, November 1991 at p.10255); *Miller, A. et al* ("Tolerance and Suppressor Mechanisms in Experimental Autoimmune Encephalomyelitis: Implications for Immunotherapy of Human Autoimmune Diseases," *FASEB, 5*:2560-2566, Aug.1991, at p. 2562, right col.); and *Miller A., et al*,"Suppressor T Cells generated by Oral Tolerization to Myelin Basic protein Suppress both In Vitro and In Vivo Immune Responses by the Release of Transforming Growth Factor β after Antigen-Specific Triggering," *P.N.A.S. (USA), 89*:421-425, January 1992, at pp. 424-425).[3] In light of the experiments described in the Weiner et al specification, it became likely that these conclusions could be extended to bystander antigens. Copies of the references cited in this Paragraph 15 are attached as Attachment 3.

QUESTION III

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[3]It was also known in 1992 that an antigen could be administered orally or nasally indefinitely as long as benefits persisted, it being well-tolerated by treated subjects.

16. I find the disclosure and admonitions of Mueller inapplicable to the claimed invention. Mueller is concerned exclusively with the concept of anergy, which is a totally different immunological concept from bystander suppression. Anergy can only be induced when an activated T-cell encounters (under certain circumstances, namely in the absence of co-stimulatory factors) the very antigen against which it is directed. I am not aware of any other antigen that can induce anergy for such a T-cell. Accordingly, if it is desired to suppress an immune response by anergizing the T-cells that mediate it, each such T-cell must be exposed to the very antigen that it recognizes. It is therefore necessary to know the specificity of each T-cell in order to administer the proper antigen. It is also necessary to ensure the absence of co-stimulation. If co-stimulation is present, an aggressive immune response can ensue instead of immune regulation; hence the extreme caution permeating the statements in Mueller.

17. Anergy is thus totally different from bystander suppression which can proceed by administration of an antigen that does not constitute a T-cell target of the aggressive response to be suppressed, and without fear of co-stimulation. Moreover, bystander suppression takes place because the elicited regulatory T-cells which recognize the administered antigen secrete nonspecific immunosuppressive factors, such as TGF-β , which suppress immune responses at the locus of their

secretion without regard to the specificity of the activated T-cells that may be found in such locus. For these reasons, the statements in Mueller, while applicable to anergy, are inapplicable to bystander suppression.

18. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Dated: October 24th, 2000

Matthias G. Von Herrath

Tolerance and suppressor mechanisms in experimental autoimmune encephalomyelitis: implications for immunotherapy of human autoimmune diseases

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ABSTRACT **Recent advances in understanding antigen recognition at the level of the trimolecular complex have provided new approaches for selective immunotherapy. Many of these approaches have been applied successfully to the animal model experimental autoimmune encephalomyelitis, and some are being tested in the human disease multiple sclerosis. In addition, new approaches utilizing nonspecific modulation of immune function are being explored in animals and humans. Immunospecific therapy in autoimmune diseases will ultimately be based on understanding how the normal immune system maintains unresponsiveness to self and how this state of self-tolerance is broken. Strategies for specific immune intervention in human diseases based on components of the trimolecular complex will have to take into account the polymorphism of the major histocompatibility complex in humans and the degree of heterogeneity among autoimmune T cells that react with an autoantigen.—Miller, A.; Hafler, D. A.; Weiner, H. L. Tolerance and suppressor mechanisms in experimental autoimmune encephalomyelitis: implications for immunotherapy of human autoimmune diseases. *FASEB J.* 5: 2560–2566; 1991.**

Key Words: autoimmunity · immunotherapy · experimental autoimmune encephalomyelitis · tolerance

THE IMMUNE SYSTEM IS CONFRONTED BY a variety of molecules, among which it must discriminate between self and non-self. Immunological tolerance is the acquisition of unresponsiveness to self-antigens, and as such is essential for preservation of the integrity of the host. Understanding the mechanisms of tolerance has become a central issue in immunology, especially in relation to ways in which breakdown of self-tolerance that results in autoimmune diseases can be prevented or reversed.

Self-tolerance is not preprogrammed in the germline but is acquired somatically by mechanisms that delete or inactivate autoreactive clones. Mechanisms of self-tolerance to T cells include clonal deletion, clonal anergy, and active suppression (**Table 1**). Since the first description of acquired immunological tolerance (1), a variety of experimental systems have been used to define the extent to which each of these mechanisms contributes to developing and maintaining self-tolerance. There is direct evidence that clonal deletion of autoreactive T cells within the thymus is one of the primary mechanisms responsible for maintaining immunological self-tolerance (2, 3). Clonal deletion occurs at the $CD4^+CD8^+$ double-positive or single-positive stage of T cell development in the thymus, and involves participation of the CD8 or CD4 molecules in recognition of major histocompatibility complex (MHC)[1] class I or class II molecules. Nonetheless, clonal deletion does not remove all autoreactive T cells, especially for tissue-specific antigens (e.g., components of the eye, brain, or testes), which are not expressed in the thymus. Immunological unresponsiveness to sequestered tissue antigens such as brain antigens may be attributed partially to the location of these self-antigens behind natural anatomical barriers or to the lack of expression of class I or class II MHC molecules in the target organ. Furthermore, autoreactive T cells may be down-regulated or suppressed either in the periphery or in the thymus by encountering antigen in the absence of MHC or another costimulatory signal (or signals) from antigen-presenting cells (APCs) leading to functional inactivation but not deletion of autoreactive T cells (clonal anergy) (4–6). Finally, autoreactive cells may be actively suppressed by regulatory or suppressor T cells that inactivate or lyse autoreactive cells in a manner that is not yet precisely defined (7–11).

EAE AS A MODEL FOR ORGAN-SPECIFIC AUTOIMMUNE DISEASE

Elucidation of mechanisms underlying human diseases and design of immunotherapy is often facilitated through the study of animal models. Because of similarities in both clinical expression and pathology, experimental autoimmune encephalomyelitis (EAE) has been used as the primary model for multiple sclerosis (MS), a human demyelinating disease of the central nervous system (CNS) of presumed autoimmune etiology (12). Experimental autoimmune encephalomyelitis is an inflammatory disease of the CNS manifest in rodents either as an acute or chronic relapsing process. Immunization of animals with myelin basic protein (MBP) plus complete Freund's adjuvant stimulates a population of autoreactive $CD4^+$ T cells that migrate to the CNS and cause disease. Myelin-specific $CD4^+$ T cell lines and clones can be generated, and upon adoptive transfer will cause disease in naive recipients (13). Myelin basic protein has been the CNS encephalitogen most extensively studied, although more recently it has become clear that proteolipid protein (PLP) is also a major CNS encephalitogen (14). The encephalitogenic epitopes of MBP differ among animal strains and are re-

[1]Abbreviations: MHC, major histocompatibility complex; APCs, antigen-presenting cells; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; CNS, central nervous system; MBP, myelin basic protein; PLP, proteolipid protein; MSCH, mouse spinal cord homogenate; TCR, T cell antigen receptor; CFA, complete Freund's adjuvant.

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TABLE 1. *Mechanisms of immunologic tolerance*

	References
Clonal deletion	
Negative selection by MHC molecules during thymic development	2, 3
Clonal anergy or functional inactivation	
Lack of costimulatory signal by cell which presents antigen resulting in an inability to make IL 2	4-6
Active suppression	
Regulatory T cells with cytotoxic activity or that suppress via soluble factors or noncytolytic cell contact	7-11

stricted by MHC class II molecules, predominantly I-A, and in a few instances I-E (15, 16).

FACTORS ASSOCIATED WITH AUTOIMMUNITY AND SUSCEPTIBILITY TO EAE

Certain animal strains are resistant to development of EAE, and susceptibility is linked to MHC genes (17, 18). A similar situation occurs in humans in which certain HLA types are associated with specific autoimmune diseases, e.g., multiple sclerosis (DR2, DQW1), diabetes (DR3, DR4), and rheumatoid arthritis (DR4) (19). The association between autoimmune diseases and particular MHC alleles suggests that T cells restricted by MHC gene products are involved in the disease process. Further support for the role of T cells in autoimmune diseases is the association with certain T cell receptor β-chain alleles (20).

The mechanism by which MHC molecules are associated with susceptibility to autoimmune diseases is unclear, but probably relates to preferential presentation of autoantigens to T cells and perhaps preferential stimulation of certain classes of T cells. However, resistance or susceptibility to EAE and other autoimmune processes is clearly a multigenic process, with genes outside the MHC complex influencing susceptibility (21).

It has been shown that astrocytes can present myelin basic protein to encephalitogenic T cell lines in vitro (22), and that bone marrow-derived perivascular microglial cells in the CNS present antigen in vivo (23). In vivo studies demonstrated the requirement for histocompatibility between invading encephalitogenic T cells and perivascular microglial cells for induction of EAE (23). Recent observations suggest that among immunological activities of the cells of the CNS, resistance or susceptibility to autoimmune processes may be related in part to the ability of brain endothelia or glia cells from susceptible strains of rats or mice to express sufficient levels of MHC to stimulate autoreactive T cells (24). These differences in stimulating capacity between species appear tissue-specific as non-glial cells from various strains had equivalent abilities to stimulate T cell lines. These observations raise the possibility that MHC antigens on glial and non-glial tissue are different, and such a difference could be important in determining T cell-mediated, organ-specific autoimmunity. Aberrant MHC expression has also been postulated to play a role in endocrine autoimmunity (25). Furthermore, in addition to presentation of antigen to the T cell receptor by antigen-presenting cells, costimulatory signals are required for T cell triggering, and T cell anergy can develop if antigen is presented to T cells in the absence of such signals (4-6). It is possible that antigen-presenting cells in the CNS constitutively anergize autoreactive T cells that migrate to the CNS by not being able to provide the necessary costimulatory signals.

EAE is induced by injection of MBP in adjuvant. The inducing events in a spontaneously occurring autoimmune disease such as multiple sclerosis are unknown. Presumably, the immune system is triggered by environmental factors such as viruses and bacteria. Possible mechanisms include infection of CNS tissue by a virus with release of autoantigen (26), molecular mimicry in which portions of a virus are homologous to CNS proteins (27, 28), or general activation of the immune system through cytokines such as γ-interferon, which are released in association with viral infection (26) (**Table 2**). Multiple sclerosis patients with an increased number of clinically evident viral infections have more attacks (29), and treatment of MS with γ-interferon made the disease worse (30).

CHRONIC RELAPSING EAE (CR-EAE)

EAE may be an acute, self-limiting process or a chronic, relapsing disease. The development of chronic as opposed to acute EAE relates to a number of factors, including the animal strain injected and the nature of the inoculation. In the Lewis rat, subcutaneous or intradermal injection of MBP plus adjuvant leads to an acute paralytic disease 10-17 days after injection, with recovery. Recovered animals are resistant to subsequent induction of EAE. Postrecovery suppressor cells of the $CD4^+$ phenotype that inhibit antigen-specific production of IFN-α by encephalitogenic T cells appear related to the recovery of Lewis rats from acute EAE and to the inability to reinduce EAE by active immunization in recovered animals (32). However, these Ag-specific suppressor cells do not inhibit disease after passive transfer of activated MBP-specific T cells, which suggests that they act at the afferent rather than the efferent phase of the disease. The existence of postrecovery suppressor cells raises the theoretical question of whether in humans natural resistance to autoimmune processes is acquired. It is possible that humans

TABLE 2. *Factors associated with autoimmune diseases*

	References
Genetic susceptibility	
Association of autoimmune diseases with MHC alleles	19
Non-MHC genes involved in disease susceptibility	20, 21
Triggering events	
Infection or damage to target organ with release or alteration of autoantigens	26
Molecular mimicry or cross-reactivity between virus or bacteria and self protein	27, 28
Abberant expression of MHC moleculesl by local antigen-presenting cells in target organ with stimulation of autoreactive T cells	24-25
Non-antigen-specific activation of the immune system by cytokines released after systemic infection	29, 30
Drugs	31
Dysregulation of the immune system	
Defective generation of suppression	36
Altered neuroendocrine regulation	33-35

become naturally protected against autoimmune processes by having self-limited or transient subclinical autoimmune attacks, and autoimmune disease develops when there is a defect in developing such resistance. Spontaneous recovery of Lewis rats from EAE depends on regulation of the immune system by endogenous adrenal corticoids (33). A defective hypophysis-pituitary-adrenal axis response to inflammation results in low levels of endogenous corticosteroids and may contribute to susceptibility of the Lewis rat to EAE and other autoimmune diseases such as streptococcal cell wall arthritis (34).

In multiple sclerosis and other autoimmune diseases such as rheumatoid arthritis, defects in antigen nonspecific immunoregulation have been identified, and such defects could contribute to generation of a relapsing/chronic immune response (36). A mild relapsing form of EAE can be triggered in Lewis rats when they are injected with whole spinal cord homogenate (37). CR-EAE is easily produced in the mouse either by injection of white matter homogenate in complete Freund's adjuvant (CFA) or the transfer of MBP reactive T cell clones (38). Immunoregulatory mechanisms associated with CR-EAE are poorly understood. One important principle related to relapsing disease is that the myelin antigen or the determinant on the myelin antigen associated with the chronic disease may be different from the determinant or antigen that induces the initial attack (39). For example, relapsing disease induced by MBP or an MBP clone might depend on induction of a cellular or humoral immune response to another myelin antigen, such as proteolipid protein (40), myelin oligoclondrocyte glycoprotein (41), or other myelin antigens. If this is true, it has important implications for designing therapy related to antigen-specific modulation of any component of the trimolecular complex.

IMMUNOTHERAPY BASED ON SPECIFIC MODULATION OF THE TRIMOLECULAR COMPLEX

Antigen recognition by T cells involves interaction of three components: antigen, the T cell receptor, and MHC. To down-regulate antigen-specific immune responses, each component is a potential target for immunotherapy (**Fig. 1**).

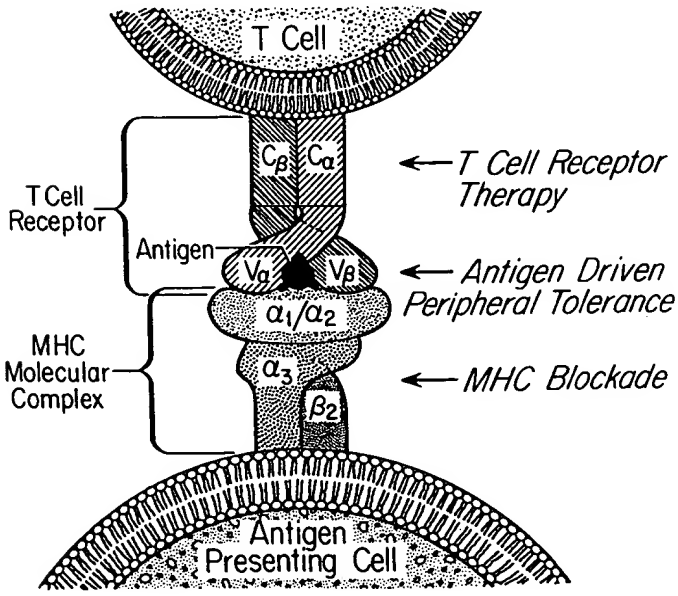

Figure 1. The trimolecular complex as a target for immunotherapy.

Indeed, successful antigen-specific immunotherapy in EAE has been achieved by using or affecting the function of each component of the trimolecular complex (**Table 3**). Some of these approaches are being tested in clinical trials in MS and other autoimmune diseases.

ANTIGEN-DRIVEN PERIPHERAL TOLERANCE

When the immune system is confronted by a foreign antigen, two pathways are possible: tolerance or immunity. Whether tolerance or immunity develops depends on many factors, including the route of administration, whether adjuvants are used, and the concentration and physical state of the antigen. A variety of methods have been used to suppress EAE using antigen-driven tolerance, including administration of antigen intravenously, in incomplete Freund's adjuvant, in liposomes coupled to autologous splenocytes, and orally (42–49). We have been investigating oral tolerance as a method to down-regulate both experimental and human autoimmune disease because of its inherent clinical applicability.

Oral administration of antigens was the first demonstration of immune tolerance and was reported in 1911 by Wells, who found that guinea pigs fed hen's egg protein lose the ability to develop anaphylactic sensitivity to the ingested protein (50). In 1946, Chase reported suppression of responses to haptens after oral administration (51). Oral immunization induces local B cell responses in the gut mucosa and at the same time tolerizes for systemic administration of the same antigen. Although the exact mechanism of suppression associated with oral tolerance is not defined, many experiments demonstrate that active suppression mediated by T cells occurs after oral administration of antigen (52). Antigen feeding affects both humoral and cellular immune responses but has more profound effects on cellular immunity.

Some investigators have suggested that after antigen is orally administered, $CD4^+$ suppressor/inducer cells are generated in Peyer's patches, and these cells then migrate to mesenteric lymph nodes and the spleen where they down-regulate systemic immune responsiveness by inducing $CD8^+$ suppressor-effector cells (53). Furthermore, it has been reported that intestinal epithelial cells may preferentially activate $CD8^+$ suppressor T cells (54).

Although oral tolerance has been studied for many antigens, it has only recently been applied to the study of autoimmune diseases. We and others have demonstrated that orally administered myelin basic protein and myelin antigens suppress acute and chronic-relapsing EAE (9, 44–46), S-Ag suppresses experimental autoimmune uveitis (55), and collagen II suppresses both collagen and adjuvant-induced arthritis (56–58). Suppression of EAE is related to the generation of antigen-specific $CD8^+$ T cells that can adoptively transfer protection against EAE (9) and act via the release of the suppressive cytokine TGF-β after being triggered by antigen (59). We have also found that orally administered nonencephalitogenic fragments of MBP can suppress EAE. Thus, antigen-driven peripheral tolerance may involve suppressor epitopes that are different from immunodominant epitopes on the antigens that trigger $CD4^+$ cells. This phenomenon has previously been shown in the HEL system (60) and in the EAE model (61). It is not clear why certain epitopes of a protein may have suppressive properties. Nonetheless, these findings raise the possibility that nonencephalitogenic fragments of an autoantigen such as MBP could be used to treat a disease such as MS, although different suppressor epitopes may be active in different individuals. A clinical trial

TABLE 3. *Immunotherapy based on specific modulation of the trimolecular complex*

	References
Antigen-driven tolerance	
Oral tolerance	9, 44–46, 55–58
Antigen coupled to autologous cells	47–49
Anti-TCR immunotherapy	
Anti-TCR monoclonal antibodies	67, 68
T cell vaccination	65, 66, 75
T cell receptor peptides	70, 71
MHC blockade	
MHC-blocking peptides	76–78
Anti-MHC class-II antibodies	79, 80

is currently in progress at our institution in which a myelin preparation is being orally administered to MS patients.

Another method to induce antigen-driven peripheral tolerance both in vitro and in vivo is by administration of antigen coupled to syngeneic lymphoid cells. In the EAE model, injection of mouse MBP-coupled splenocytes 7 days before immunization with mouse spinal cord homogenate (MSCH) prevents induction of acute EAE in SJL/J mice (47), and this approach also suppresses MSCH-induced CR-EAE (48). Neuroantigen-specific tolerance has been used to determine the relative role of MBP and PLP in MSCH-induced CR-EAE (49). It has been found that tolerization with PLP, not MBP-coupled spleen cells, prevented disease in these animals. These experiments demonstrate that antigen-driven tolerance can be used to determine which autoantigens are important in an autoimmune disease in which multiple antigens are potentially involved. In this regard we recently demonstrated that oral administration of type II collagen suppresses adjuvant arthritis (58), which suggests an important role for autoimmunity to CII in this model. In a similar fashion, a clinical trial in which antigen-driven tolerance is undertaken will in itself be a clinical experiment that will help define the degree to which immunity to particular autoantigen plays a pathogenic role in the disease.

A synthetic copolymer (Cop 1) that competes with MBP for binding to MBP reactive clones (62) suppresses EAE (63), and in a pilot trial subcutaneous administration demonstrated positive effects in patients with early, relapsing MS (64) although no effect was observed in the chronic form. The exact mechanism of action of Cop 1 in vivo is unclear. It was initially designed to generate antigen-driven tolerance due to its cross-reactivity to myelin basic protein, and is reported to induce MBP-specific suppressor cells (63). It is postulated to also act by specific inhibition of BP-specific effector cells.

ANTI-T CELL RECEPTOR THERAPY

EAE is mediated by $CD4^+$ T cells specific for MBP or PLP. Cohen and co-workers (65) were able to suppress EAE by injecting MBP-specific T cell lines that were attenuated by irradiation, pressure, or glutaraldehyde treatment before immunizing animals with MBP plus CFA. The effect was for the most part antigen-specific in that injection of lines specific for another antigen had a minimal effect. This treatment has been termed T cell vaccination, and the presumed mechanism of action is an anticlonotypic or antiidiotypic response against the T cell antigen receptor (TCR) or other structures on the injected line or clone (66). Subsequently, it has been found that there is conservation of TCR among encephalitogenic T cells, even in different animal models of EAE, despite known differences in MBP-epitope specificity and differences in major histocompatibility complex (MHC) restriction (67–69). These findings provided a more defined target for immunotherapeutic intervention. Two groups have reported successful treatment of EAE in the Lewis rat by immunizing with peptides comprising specific variable regions of the Vb8.2 T cell receptor (70, 71). Another successful approach has been the use of monoclonal antibodies directed against TCR structures used by encephalotogenic clones (67, 68). Nonetheless, anti-idiotypic antibodies directed against the T cell receptor of an MBP-specific T cell hybridoma have been reported to either suppress or enhance EAE (69). For anti-T cell receptor therapy to be successful in human disease, there must be limited heterogeneity of TCR usage by pathogenic autoreactive T cells. We have recently demonstrated restricted TCR usage for MBP clones from MS patients that recognize the immunodominant region of MBP, raising the possibility of T cell receptor therapy in MS (72). Furthermore, oligoclonal T cells have been described both in MS (73) and rheumatoid arthritis (74). Pilot trials of T cell vaccination using T cells clones from spinal fluid or joints in human autoimmune disease such as MS and rheumatoid arthritis are currently in progress (75). Other investigators are testing T cell receptor peptides in MS. Nonetheless, it is likely that with further study TCR usage in the recognition of autoantigens in autoimmune models and human diseases may not be strictly limited.

MHC-BLOCKING PEPTIDES AND ANTI-MHC MONOCLONAL ANTIBODY THERAPY

A third approach for immune intervention at the level of the trimolecular complex relates to the fact that autoantigens must be presented by MHC molecules and that there is an association between autoimmune diseases and particular MHC alleles (19). Based on recognition of the MBP peptide (P1-11), acetylated at its NH_2 terminal, as the immunodominant epitope that stimualtes encephalitogenic T cells in H-2^u strain mice, it was possible to design peptides that competed for recognition of MBP at the level of antigen presentation without interacting with TCR to block T cell activation (76). By substitution of a single amino acid (lysine to alanine at position 4 of Acl-11), it was possible to generate peptides with increased affinity for the I-A^u binding determinant. The same peptides substituted with other residues (glutamine to alanine in position 3) are not recognized and do not activate encephalitogenic T cells. Immunization of mice with both the analog peptide and the encephalitogenic peptide suppressed EAE compared with animals given the encephalitogenic peptide alone (77). Peptide blocking of MHC has also been shown for PLP-induced EAE by peptide 139-151 (78). To be effective, the MHC blocking peptide must compete with endogenous peptides for the MHC binding site and therefore must be continually supplied to avoid induction of the autoimmune response, which may not be feasible clinically.

EAE as well as other autoimmune diseases have been treated effectively by administration of anti-Ia monoclonal antibodies (79, 80). The mechanism of action of anti-Ia immunotherapy is unclear. Such therapy was designed to inhibit MHC presentation of Ag to T cells and offer the advantage of allelic-specific therapy, suppressing autoimmune responses linked to certain alleles without inducing global immune suppression. Investigators have also reported generation of T cells that adoptively transfer suppression after anti-Ia therapy (80, 81).

IMMUNOTHERAPY BASED ON MODULATION OF NONSPECIFIC IMMUNE FUNCTION (TABLE 4)

Monoclonal antibodies against T cell surface markers

Monoclonal antibodies that recognize T cell surface determinants not related to the TCR have been used in animals as immunosuppressive agents to reverse experimental models of autoimmune disease (82, 83). $CD4^+$ T cells are the primary disease-inducing component in EAE. Both anti-pan T cell antibodies and anti-CD4 antibodies suppressed acute, and in some instances chronic, EAE (84). Another approach has been to target activated T cells in EAE (85), and anti-IL 2 receptor antibody has been shown to be effective in the NOD mouse model of diabetes (86). Interleukin 2 linked to diptheria toxin is another method to target activated cells (89). Pilot trials with murine monoclonal antibodies directed against T cell-surface antigens (CD4, CD2, and anti-T-12) have been reported in MS patients (87, 88). Immunologic effects were noted, but human anti-mouse responses prevented continued administration of these monoclonal antibodies for a chronic disease such as multiple sclerosis (88). Chimeric antibodies are expected to have less antigenicity, and thus may partially obviate the limitation of mouse mAb due to the human response against the mouse mAb constant regions. There are preliminary reports of positive effects of anti-CD4 antibodies in rheumatoid arthritis, and trials of chimeric anti-CD4 antibodies in MS patients are currently in progress. Another possible approach includes the use of monoclonal antibodies directed against adhesion molecules.

Other forms of therapy

Cytokines play an important role in the generation of immune responses, and a variety of approaches related to moderation of cytokine function or use of cytokines to alter immune reactivity are possible. As mentioned previously, MS was exacerbated by treatment with γ-interferon, presumably related to up-regulation of class II (30), and MS patients with increased numbers of viral infections have increased disease activity (29). Currently, there are multicenter trials utilizing β-interferon in MS based on the antagonistic effects of β-interferon on γ-interferon-induced Ia expression (90). Soluble IL 1 receptors are also being used to suppress immune responses (89).

Migration of activated lymphocytes into the target organ is the prerequisite for initiation of cell-mediated autoimmune responses. We have found rapid trafficking of in vivo-labeled T cells from peripheral blood to the cerebrospinal fluid in MS (91). It has been shown that traffic of T cells to target tissue is related to activation of the heparanase that degrades the heparan sulfate component of the subendothelial extracellular matrix. Expression of this heparanase and heparanase-dependent T cell traffic was shown to be inhibited in vitro and in vivo by heparinoid molecules, and suppression of experimental autoimmune diseases was demonstrated in animals by treatment with low doses of heparin (92). We performed a pilot trial of low-dose heparin (100–500 units/day) in chronic progressive MS but observed no positive effects (93).

Finally, chemical immunosuppression with drugs such as cyclophosphamide have been shown to be of benefit in some patients with MS (94) and in lupus nephritis. Methotrexate is efficacious in rheumatoid arthritis. Because of the multiple mechanisms involved in autoimmune responses, intermittent use of such drugs may be required even in the context of more specific modulation of the immune system discussed previously. FJ

TABLE 4. *Immunotherapy based on modulation of nonspecific immune function*

	References
Monoclonal antibodies directed against T cells	
Anti-CD4	82–84, 88
Anti-CD2	88
Down-regulation of activated cells	
Anti-IL 2 receptor monoclonal antibodies	85, 86
IL 2 toxin	89
Cytokine therapy	
β-Interferon	90
Soluble IL 1 receptors	89
Alteration of lymphocyte migration	91–93
Chemical immunosuppression	94

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Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin

(diabetes/tolerance/autoimmunity/immunotherapy/insulin)

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ABSTRACT **Nonobese diabetic (NOD) mice spontaneously develop an autoimmune form of diabetes associated with insulitis. A number of immunomodulatory therapies have been investigated as a treatment for the disease process. Oral administration of the autoantigens myelin basic protein and collagen type II suppresses experimental models of encephalomyelitis and arthritis. We have now found that oral administration of insulin delays the onset and reduces the incidence of diabetes in NOD mice over a 1-year period in animals administered 1 mg of porcine insulin orally twice a week for 5 weeks and then weekly until 1 year of age. As expected, orally administered insulin had no metabolic effect on blood glucose levels. The severity of lymphocytic infiltration of pancreatic islets was also reduced by oral administration of insulin. Furthermore, splenic T cells from animals orally treated with insulin adoptively transfer protection against diabetes, demonstrating that oral insulin administration generates active cellular mechanisms that suppress disease. These results show that oral insulin affects diabetes and the pancreatic cellular inflammatory process in the NOD mouse and raise the possibility that oral administration of insulin or other pancreatic autoantigens may provide a new approach for the treatment of autoimmune diabetes.**

Type I diabetes or insulin-dependent diabetes mellitus (IDDM) is thought to be an autoimmune disease in humans (1–3). The nonobese diabetic (NOD) mouse spontaneously develops IDDM that has many immunological and pathological similarities to human insulin-dependent diabetes. The autoimmune nature of the disease is suggested by the lymphocytic infiltration of the islets of Langerhans, which precedes the destruction of insulin-producing beta cells (4). As such, the NOD mouse has served as one of the primary models for IDDM and a model in which new approaches for immunotherapy have been investigated.

A variety of immunomodulatory treatments have been studied in the NOD mouse. In general, treatments that affect T-cell function or are immunosuppressive have been effective, such as neonatal thymectomy and *in vivo* treatment with anti-CD4 monoclonal antibody and cyclosporine A (5–7). A major impetus behind such studies has been to develop approaches that may be utilized to treat human IDDM. Clinical trials in humans have demonstrated that antigen nonspecific immunosuppression with drugs such as cyclosporine A and azathioprine can affect beta-cell destruction after diabetes onset, but such therapy is not curative and is associated with drug-related toxicities (8, 9). The ability to identify patient populations at risk for diabetes (10, 11) makes the development of disease-specific nontoxic forms of therapy that can be administered to prediabetics to prevent or reduce the incidence of diabetes a major therapeutic goal.

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We have been investigating antigen-driven peripheral immune tolerance as a means to suppress autoimmune processes, using the oral route of antigen exposure. Orally administered antigen stimulates the immune system in a physiologic fashion and has long been recognized to produce systematic immunologic hyporesponsiveness or tolerance (12–14). We and others have found that oral administration of autoantigens suppresses animal models of autoimmunity including experimental autoimmune encephalomyelitis (EAE) (15–17), adjuvant- and collagen-induced arthritis (18–20), and experimental autoimmune uveitis (21). Oral tolerance as a means to treat diabetes is especially attractive because of its virtual lack of toxicity and its inherent clinical applicability. In addition, such therapy could be applicable to pancreatic islet transplantation. In the present report, we have found suppression of diabetes in the NOD mouse by oral administration of insulin.

MATERIALS AND METHODS

Animals. NOD mice were purchased from Taconic Farms, maintained in our animal facility, and fed regularly with Purina Mouse Chow 5015 or 5008. The animals studied in experiments in Table 1 and Fig. 4 were housed in a conventional room, and those studied in all other experiments were housed in a virus antibody-free (VAF) facility. Female NOD mice were used for all experiments except for recipients in adoptive transfer experiments.

Assessment of Diabetes. Mice were monitored for development of diabetes weekly by urinary glucose testing with test strips (Eli Lilly). Glycosuric mice were then bled to check for glycemia by using a glucose analyzer (Beckman). Diabetes was confirmed by hyperglycemia (>13.8 mM) for 2 consecutive weeks.

Antigens. Porcine monocomponent insulin was purchased from Novo Biolabs (Danbury, CT). Myelin basic protein (MBP) was prepared as described (15).

Oral Administration of Antigen. Insulin or MBP in phosphate-buffered saline (PBS; 1.7 mM KH_2PO_4/5 mM Na_2HPO_4/150 mM NaCl) was administered to mice orally through a syringe fitted with a ball-type feeding needle in a volume of 0.5 ml per mouse per feeding.

Histopathology. The animals were sacrificed by cervical dislocation, and the pancreases were taken and immediately frozen. Cryosections (3 or 4 sections per mouse) were fixed with acetone and double-stained with (*i*) biotinylated monoclonal anti-thy-1.2 antibody plus avidin–peroxidase conjugate and (*ii*) monoclonal anti-beta-cell antibody (A2B5) plus alkaline phosphatase-conjugated anti-mouse IgM. The degree of insulitis was scored blindly by two independent observers using a semiquantitative scale ranging from 0 to 4: 0, normal islet with no sign of T-cell infiltration; 1, focal peri-islet T-cell infiltration; 2, more extensive peri-islet infil-

Abbreviations: NOD, nonobese diabetic; IDDM, insulin-dependent diabetes mellitus; MBP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; VAF, virus antibody-free.

on but with lymphocytes less than one-third of the islet area; 3, intraislet T-cell infiltration in one-third to one-half of the islet area; 4, extensive intraislet inflammation involving more than half of the islet area.

Adoptive Transfer of Diabetes and T-Cell Depletion. The adoptive transfer experiments were carried out by the method of Wicker *et al.* (22) with slight modifications. Briefly, donor splenocytes were prepared from newly diabetic female animals (diagnosed within 14 days), resuspended in Hanks' balanced salt solution (HBSS), and injected i.v. through the retroorbital plexus (1×10^7 cells per recipient) to 7-week-old male NOD mice, which were irradiated with 770 R from a ^{137}Cs source 24 hr prior to the transfer. Five-million modulator cells from insulin-fed or control-fed animals were cotransferred with splenocytes from newly diabetic animals into male recipients. For T-cell depletion, splenocytes from insulin-fed animals were incubated with anti-thy-1.2 monoclonal antibody (diluted 1:200; from Accurate Chemicals, Westbury, NY) at a concentration of 2×10^7 cells per ml, at room temperature for 60 min, followed by an incubation with Low-Tox rabbit complement (1:15; Cedarlane Laboratories, Hornby, ON, Canada) for 30 min at 37°C. Control cells were treated with complement alone. Cells were washed three times with HBSS prior to transfer. Five-million anti-thy-1.2- or complement-treated cells were cotransferred.

RESULTS AND DISCUSSION

A number of autoantigens have been identified as potential t antigens of an autoimmune attack that leads to the opment of diabetes. These include insulin, glutamic acid carboxylase (GAD), carboxypeptidase H, insulin secretory granule proteins, and heat shock proteins (23–25). To test the effect of oral administration of insulin on the development of diabetes, female NOD mice at 5 weeks of age were fed PBS or 10 μg, 100 μg, or 1 mg of porcine insulin twice weekly for 5 weeks and then weekly until the animals reached 1 year of age. There was a marked delay in the onset and a decreased incidence of diabetes in animals fed 1 mg of porcine insulin (Table 1; Fig. 1, $P = 0.02$, Kaplan–Meier analysis) with a slight effect at 100 μg. Note that the incidence of diabetes in the control group was relatively low. This may be related to the frequent handling of the animals associated with feeding and to the housing of the animals for this experiment in a non-VAF facility. To test the effect of oral insulin in animals with a higher incidence of diabetes, a second experiment was conducted in a VAF facility. In addition, a group of animals was also fed 1 mg of MBP as a control antigen. A decreased incidence of diabetes following oral insulin was observed, although the overall incidence of diabetes was higher. Specifically, the incidence of diabetes in animals at 30 weeks was as follows: 13 of 30 fed PBS, 14 of

Table 1. Suppression of IDDM in NOD mice by oral administration of porcine insulin

Feeding treatment	Diabetes incidence, %		
	6 months	9 months	12 months
Control (PBS)	20.5	44.1	49.2
Insulin in PBS			
μg	16.7	23.8	37.3
0 μg	11.1	28.5	43.8
1 mg	0*	8.0*	26.4†

Five-week-old female NOD mice (27–30 per group) were fed with various dosages of porcine insulin in PBS (control group received PBS alone) twice weekly for 5 weeks and weekly until 1 year of age. Beginning at 12 weeks of age, the mice were examined weekly for

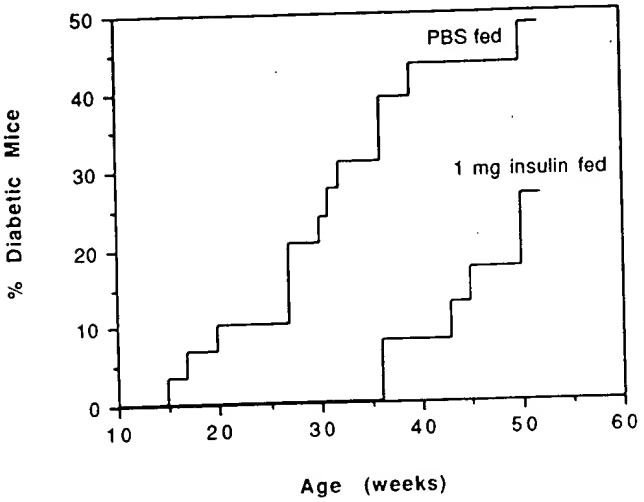


FIG. 1. Effect of oral administration of porcine insulin on IDDM in female NOD mice. Life table analysis of the control group and the group fed 1 mg of insulin from Table 1 ($P = 0.02$, Kaplan–Meier analysis).

30 fed MBP, 13 of 29 fed 10 μg of insulin, 10 of 30 fed 100 μg of insulin, and 6 of 30 fed 1 mg of insulin ($P < 0.05$ for animals fed 1 mg of insulin vs. control and animals fed MBP).

It has been reported (26) that low doses of subcutaneous insulin may affect the onset of diabetes in NOD mice. Orally administered insulin is not metabolically active, presumably because it is degraded in the stomach. Degradation of proteins in the gastrointestinal tract does not affect oral tolerance and actually may facilitate orally induced tolerance by creating small protein fragments that are better able to interact with gut-associated lymphoid tissue (27). Nonetheless, to determine whether any metabolic effects could be discerned in animals being fed 1 mg of insulin, blood glucose levels were measured in 17-week-old animals. The average blood glucose prior to the weekly insulin feeding was 7.56 mM in animals fed PBS and 7.53 mM in animals fed 1 mg of insulin. Thirty minutes after feeding, the blood glucose in animals fed PBS

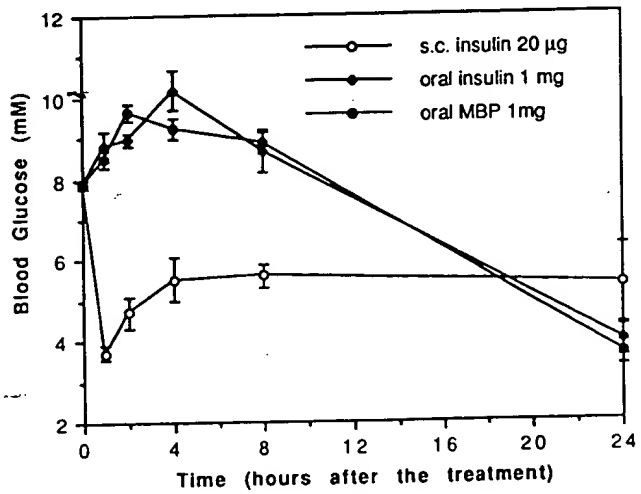
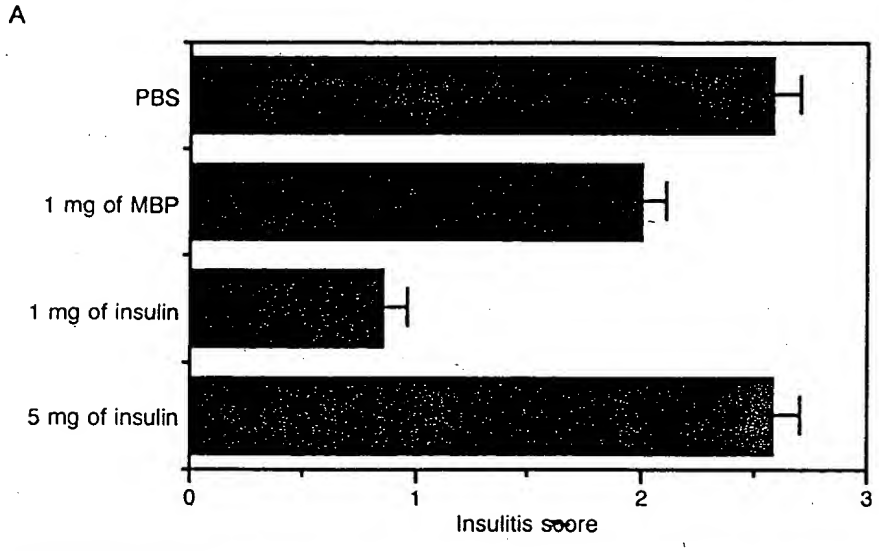


FIG. 2. Effect of oral insulin on blood glucose. Seven-week-old female NOD mice (25 mice per group) were treated orally with 1 mg of porcine insulin or 1 mg of guinea pig MBP or were injected subcutaneously with 20 μg of porcine insulin. All mice were bled before treatment, and 5 mice from each group were bled again 1, 2, 4, 8, and 24 hr after treatment. Individual plasma samples were measured in duplicate for glucose levels by using a Beckman glucose



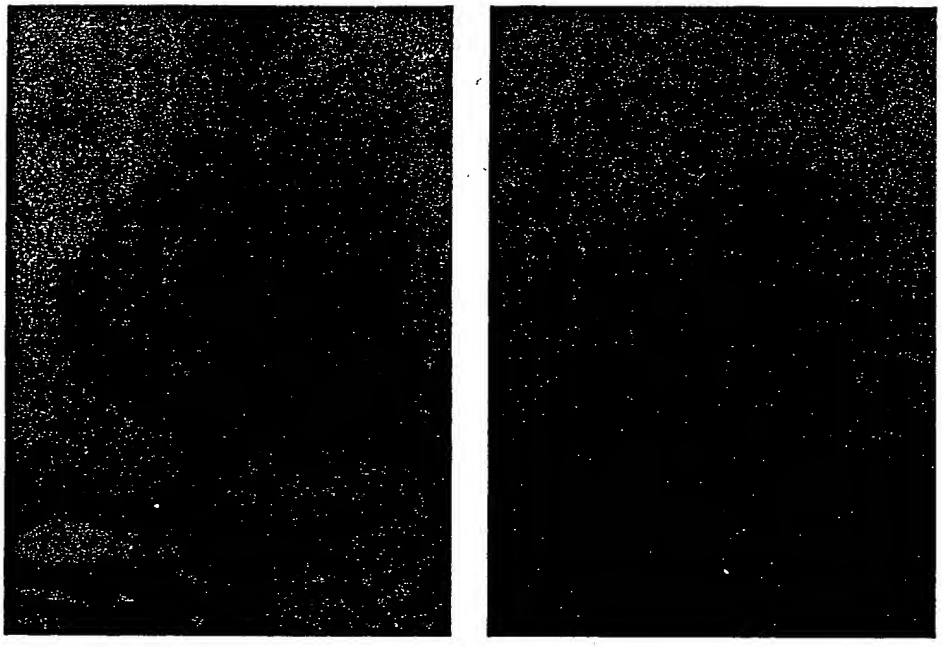


FIG. 3. Effect of feeding porcine insulin on insulitis in NOD mice. Five-week-old female NOD mice (8–10 mice per group) were fed PBS, 1 mg or 5 mg of insulin in PBS, or 1 mg of MBP in PBS twice weekly for 5 weeks. At 10 weeks of age, the animals were sacrificed, and pancreases were taken for histopathological examinations. Eight to 12 islets from each animal were scored. (*A*) Insulitis score. Data are expressed as the mean score of each group ± SEM ($P < 0.001$ for group fed 1 mg of insulin vs. group fed PBS or 1 mg of MBP). (*B*) Representative islet from control animal with pronounced lymphocyte infiltration (histopathologic score = 4). (*C*) Representative islet from animal fed 1 mg of insulin with minimal inflammation (histopathologic score = 1).

was 8.53 mM and in animals fed 1 mg of insulin was 8.63 mM. In an additional study, 7-week-old NOD mice were fed 1 mg of insulin or 1 mg of MBP. All animals were kept in a fasting state. Animals given 20 μg of subcutaneous insulin had an immediate drop in blood glucose (Fig. 2). In animals fed 1 mg of insulin or 1 mg of MBP, an increase in blood glucose was observed, perhaps related to the stress of gastric intubation, followed by a decrease in blood glucose 8 hr later as the animals were in a fasting state. Note that animals fed 1 mg of insulin for 1 year responded normally to subcutaneous insulin (data not shown). These results show that oral insulin has no metabolic effect on blood glucose either acutely or chronically.

To determine whether feeding insulin affected lymphocytic infiltration of pancreatic islets, animals in a separate series of experiments were fed 1 mg of insulin twice weekly for 5 weeks and were sacrificed at 10 weeks of age and examined for insulitis. There was a marked reduction of insulitis in animals fed 1 mg of insulin vs. those fed 1 mg of MBP (Fig. 3; 0.85 ± 0.1 vs. 1.99 ± 0.1; $P < 0.01$). Note that feeding 5 mg of insulin did not affect insulitis. A similar dose–response effect has been observed with oral tolerization to collagen in animal models of adjuvant and collagen-induced arthritis in which the suppressive effect of oral collagen was lost with increased doses (18–20). We also have observed a loss of suppression of EAE in the SJL mouse by orally administered MBP with increasing doses (28).

The majority of studies on the mechanism of oral tolerance report that active cellular suppression occurs (14). We have adoptively transferred disease protection with lymphocytes from fed animals in both the EAE and adjuvant arthritis models (16–18). To investigate whether active cellular mechanisms were associated with suppression of diabetes in the NOD mouse after oral administration of insulin, an accelerated diabetes model was utilized, in which diabetes is accelerated in young NOD mice by adoptive transfer of splenocytes from diabetic NOD donors; this provides a sensitive and more rapid assay for investigating immunomodulation of disease. Spleen cells from animals fed 1 mg of insulin, PBS, or 1 mg of MBP 5 times over 2 weeks were cotransferred with spleen cells from diabetic animals. Accelerated diabetes in NOD mice was suppressed by splenocytes from insulin-fed but not PBS- or MBP-fed animals (Fig. 4; $P < 0.001$, "logrank" test for all groups).

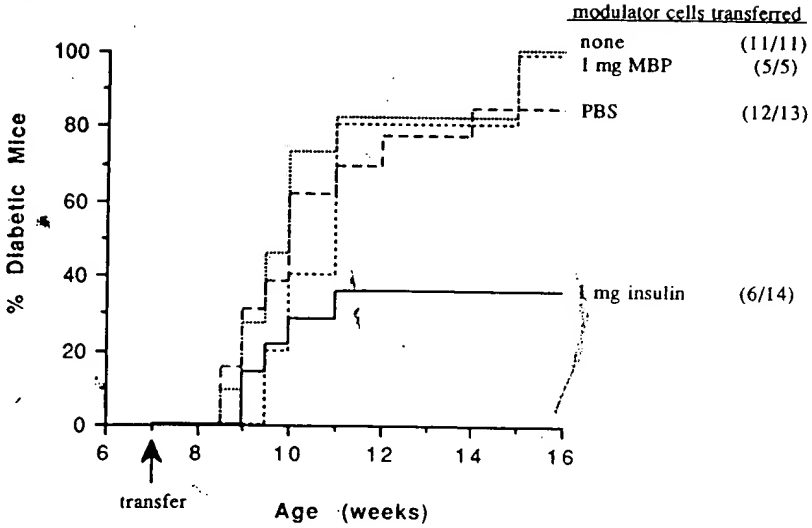


FIG. 4. Suppression of adoptively transferred diabetes by splenocytes from female NOD mice fed insulin. Modulator cells were freshly obtained from 6-week-old female NOD mice that had been fed 1 mg of insulin, 1 mg of MBP, or PBS five times over the previous 2 weeks. Ten million splenocytes isolated from female diabetic NOD mice were cotransferred with 5 million modulator cells from fed animals to 7-week-old syngeneic male recipients that had been irradiated with 770 R 24 hr earlier. The onset of diabetes in the recipients was checked twice weekly by assaying for glycosuria and confirmed by presence of hyperglycemia (>13.8 mM). $P = 0.037$ (logrank test) for all groups; $P = 0.021$ for animals fed insulin vs. those fed PBS.

To determine whether the suppression was T cell dependent, T cells were depleted from splenocytes of insulin-fed animals prior to adoptive transfer. Eight weeks after transfer, the incidence of accelerated diabetes in animals receiving no modulators was 10/11; in animals receiving complement-alone-treated modulators from insulin-fed animals was 2/10, and in animals receiving T cell-depleted modulators from insulin-fed animals was 9/10 ($P = 0.02$). Others have reported suppression of accelerated disease with transfer of 20×10^6 ...leen cells from nondiabetic 8-week-old animals (29). We ... not observe protection by spleen cells from control ...nals with 5×10^6 cells transferred.

The effects we observed in the NOD mouse are not related to nonspecific suppressive effects of orally administered insulin as oral administration of 1 mg of insulin had no effect on the development of EAE in the SJL mouse or on cellular proliferative response to concanavalin A or lipopolysaccharide (data not shown). In other studies of oral tolerance in autoimmune models, we also found disease protection to be antigen and disease specific. Thus, the antigens we have used for oral tolerization, MBP, collagen type II, and S antigen suppress EAE, adjuvant arthritis, and experimental uveitis, respectively, without affecting the other diseases. Species-specific autoantigens are not a requisite to induce oral tolerance as we have found suppression of EAE in the Lewis rat with bovine MBP.

Although we have shown suppression of diabetes and insulitis in the NOD mouse by oral administration of insulin, the role of autoimmunity to insulin in the development of diabetes in the NOD mouse and in man remains to be defined. Anti-insulin antibodies are found in both NOD mice and patients with type I diabetes (30). In patients, anti-insulin autoantibodies can be found prior to the onset of insulin therapy, are HLA-DR4-associated, and are correlated with the rate of disease progression (31, 32). Cellular reactivity to insulin occurs in man and has been reported to be of increased frequency in prediabetic individuals (33). Cellular immunity to insulin has not been extensively studied in the NOD mouse, and in initial experiments we have not found cellular immune responses to insulin as measured by thymidine incorporation ...he spleen or lymph nodes of NOD mice, though further ...tigations are required in this area using more sensitive ...ys and studying cells isolated from the pancreas.

Adoptive transfer experiments demonstrate that transferable active suppression of diabetes in the NOD mouse by splenic T cells is generated by oral administration of insulin. Recent studies from our laboratory suggest that the T cells that adoptively transfer suppression of experimental autoimmune encephalomyelitis following oral administration of MBP are triggered in an antigen-specific fashion but mediate their effect by the release of the antigen-nonspecific suppressor cytokine transforming growth factor β (TGF-β) in close proximity to effector cells (34). We have termed this mechanism "antigen-driven bystander suppression" (35). Thus, it is possible that insulin is not a pathogenic autoantigen in the NOD mouse but that the regulatory cells generated in the gut by feeding insulin migrate to the pancreas and are triggered by insulin to release TGF-β, which down-regulates the local inflammatory processes in the pancreas. Further investigations are required to determine whether oral administration of insulin affects diabetes in the NOD mouse by suppression of anti-insulin autoimmunity or by the aforementioned antigen-driven bystander suppression mechanism.

It remains to be determined whether oral administration of other islet cell-specific antigens such as glutamic acid decarboxylase, carboxypeptidase H, heat shock proteins, or secretory granule proteins can also suppress diabetes in the NOD mouse. For application to human disease states, we have found that oral administration of autoantigens suppresses both established EAE and adjuvant arthritis, demonstrating the ability to effect an ongoing immune response (18, 36).

Although our data clearly demonstrate amelioration of diabetes in the NOD mouse by oral administration of insulin, protection is not complete. We have observed that adjuvants such as lipopolysaccharide, when given orally, enhance the protective effects of oral tolerance to MBP in the EAE model (37). In addition, after week 10 of the NOD mouse, insulin was administered once per week. Thus, the use of tolerogenic adjuvants to enhance suppression, or more frequent dosing schedules, may lead to more complete prevention of diabetes. Additionally, it may be that oral administration of more than one or a different pancreatic target antigen will further enhance protection.

One of the primary goals for the immunotherapy of autoimmune diseases is to find nontoxic antigen-specific therapies that can be administered early in the course of the disease. Our results in the NOD mouse model of diabetes raise the possibility that orally administered insulin and/or other pancreatic antigens could provide a new approach for the prevention and treatment of autoimmune diabetes in man.

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TABLE OF CONTENTS

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Current grant support

JDF Career Development Award, July 1996, for 4 years *(DNA vaccination to treat IDDM/mechanism of β-cell destruction)*	75,000$/year
NIH (NIDDK) R29 grant June 1996, for 5 years *(Oral tolerance to treat IDDM, cytokines in diabetes pathogenesis)*	72,000$/year
NIH (NIAID) R01 grant October1998, for 3 years *(Regulatory lymphocytes to treat IDDM, cytokine signaling pathways in IDDM, costimulation blockade to treat IDDM)*	163,000$/year
NIH (AG) Project on Program project grant, December 1998, for 5 years *(effect of age on cytokines and autoreactive cells in autoimmune disease)*	89,000$/year
Total:	**399,000$/year**

Awards and Fellowships

DAAD Fellowship - Gene technology - 1986
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JDF (Juvenile Diabetes Foundation) Fellowship Award - 1993-1995
ASV Travel fellowship for Int. Congress of Virology 8/96
AAI Travel fellowship for Int. Congress of Immunology 11/98

Individuals trained and recruited:

Independently:

1998-present: Urs Christen, Ph.D., Postdoctoral Fellow, supported by Swiss Nat. Foundation

1998-present: Angelika Jahreis, Postdoctoral Fellow, supported by German Leopoldina Fellowship

10-12/1998: Mette Ejrnes, Ph.D. Student from the Hagedorn Res. Inst. Denmark, supported by NovoNordisk Stipend.

1-8/1999 : Sonja Seewaldt, Ph.D. Student from the European Nat. University, Strassbourg, supported by DGFI and DAAD fellowships.

9/1999-8/2000: Ursula Möhrle, Student from the University Freiburg Medical Center, Freiburg, Germany, supported by the Carl Duisberg Foundation.

Jointly with Michael Oldstone:

1996-present: Dirk Homann, MD, Postdoctoral Fellow, supported by DAAD and SDV Stipends

1997-present: Andreas Holz, Ph.D., Postdoctoral Fellow, supported by Swiss Nat. Foundation

Professional Memberships

American Society of Virology
American Society of Immunology
International Diabetes Society
Juvenile Diabetes Foundation International
American Diabetes Association
World Affairs Council, San Diego

Study Sections:

Juvenile Diabetes Foundation, Study Section, permanent member 1999-2001
NCRR Comparative Medicine, Ad Hoc, VA Grant Reviews
NIH ad hoc reviews for the NIAID and NIEHS

Reviews for journals:

Nature Medicine
Journal of Immunology
Journal of Virology
Virology
Immunology Today
Journal of Clinical Investigation
Hepatology
Journal of Autoimmunity
European Journal of Immunology
Archives of Virology
European Journal of Immunology

Invited Major Presentations:

2/1994 - Seminar, Department of Experimental Pathology, University of Zurich, Switzerland
Host: Professor Zinkernagel

2/1994 - Faculty Seminar, Max Planck Institute for Immunobiology, Freiburg, FRG
Host: Professor Eichman

2/1995 - Faculty Seminar, Dept. of Experimental Pathology, University of Zurich,
Host: Professor Zinkernagel

2/1995 - Faculty Seminar, Max Planck Institute for Immunobiology, Freiburg, FRG
Host: Professor Hartmut Peter

6/1995 - Invited Speaker, FASEB Summer Conference on Autoimmunity in Saxton River, Vermont, USA

3/1996 - Invited Speaker, International Royal Society of Medicine (London) "Molecular Mimicry" Meeting. Host: Professor Harold Baum

6/1996 - Group Lecture, Department of Microbiology, Emory University Atlanta, USA
Host: Prof. R. Ahmed

6/1996 - Group Lecture, Department of Immunology, Yale University New Haven, USA
Host: Dr. R. Flavell

8/1996 - Invited Speaker, University of Montreal, Canada, Host: Dr. Trevor Owens

12/1996 - Faculty Seminar Series, Department of Experimental Pathology, Zurich, Host: Prof. Zinkernagel

12/1996 - Invited Plenary Speaker, International Immunology of Diabetes Congress, Canberra, Australia. Host: Professor Kevin Lafferty

12/1996 - Invited Plenary Speaker, British Society of Immunology Congress in Harrogate, UK. Host: Dr. Anne Cooke, Dr. Diego Vergani

3/1997 - Invited Plenary Speaker, German Virology Congress, Hamburg, FRG

Host: Professor Lehman-Grube

4/1997 - Invited Speaker; Barbara Davis Center for Childhood Diabetes, Denver, CO, Host: Dr. Ron Gill

7/1997 - Invited Plenary Speaker, Lessons from Animal Diabetes (LAD) Workshop, Copenhagen

3/1998 - Invited Speaker, ThymOz Conference, Heron Island, Australia, Host: Dr. R. Boyd

6/1998 - Invited Speaker, Immunology Seminar Series, Univ. of California, San Francisco, Host: Prof. Steinunn Baekkeskov

6/1998 - Invited Speaker, Main Immunology Seminar Series, University of Cleveland, OH, USA
Host: Prof. Paul Lehmann, Thomas Forsthuber

9/1998 - Invited Plenary Speaker, German Immunology Congress, Freiburg, FRG

10/1998 - Invited Speaker, Symposium on Basic Aspects of Immunology by USCHS, Washington DC

11/1998 - Invited Plenary Speaker, International Congress of Immunology, New Delhi, India

1/1999 - Invited Plenary Speaker, Keystone Conference on Mucosal Immunology, Santa Fe, NM

3/1999 - Invited Speaker, 3rd International Congress of Autoimmunity, Tel Aviv, Israel

8/1999 - Invited Speaker, International Multiple Sxlerosis Workshop, Brighton, England

9/1999 - Invited Speaker, Chilean Society of Immunology Conference, Santiago, Chile

9/1999 - Invited Speaker, AASLD Conference on Autoimune Hepatitis, Atlanta, GA

9/1999 - Invited Speaker, Pathogenic and Regulatory Cell in Demyelinating Diseases Conference, Rome, Italy

Chaired Scientific Sessions and Workshops:

12/1996 - Workshop Chair, International Immunology of Diabetes Congress, Canberra, Australia

4/1997 - Co-Chair, Keystone Conference on Tolerance and Autoimmunity, Keystone, CO, USA
Host: Polly Matzinger

6/1998 - **Invited Chair,** American Society of Virology Congress, Vancouver, B.C., Canada
Host: Sue Moyer

Other Invited Presentations:

2/1995 - Interdisciplinary Lecture, CNRS, Toulouse, France
Host: Dr. Jean E. Gairin

2/1995 - Group Lecture, NovoNordisk and University of Copenhagen, Denmark.
Host: Dr. Thomas Dyrberg

6/1995 - Group Lecture, Dept. of Cancer Biology, Harvard Medical School, Boston, MA, USA
Host: Professor Laurie Glimcher

10/1995 - Seminar, Immunology Special Interest Group, Lab. Of Cell. and Mol. Immunology, NIH, Washington DC, USA.

3/1996 - Group Lecture, NovoNordisk and Univ. of Copenhagen, Denmark.
Host: Dr. Thomas Dyrberg

3/1996 - Faculty Seminar, INSERM Paris, Hopital Necker,
Host: Prof. J.F. Bach

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Bot, A., Coon. B., and **von Herrath, M.G**. Overexpression of IL-4 in the lung interferes with priming and recruitment of influenza specific memory CTL. *Virology, in press, 2000*

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von Herrath, M.G., Berger, D., Wolfe, T., Hoimann, D., Sette, A., and M. Oldstone Vaccination to treat persistent infection. *Virology in press, 2000*

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Sevilla, N., D. Homann, **M.G. von Herrath**, F. Rodriguez, S. Harkins, J.L. Whitton and M.B.A. Oldstone. Virus-induced diabetes in a transgenic model: Role of cross-reacting viruses and quantitation of effector T cells needed to cause disease. J. Clin. Invest., submitted, 1999.

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Published work from Ph.D. thesis:

von Herrath, M.G., G. Hasenfuss, C. Holubarsch, H. Hoffmann and H. Just. Repeated determination of left ventricles wall-thickness from mass and volume during one cardial cycle for the calculation of left ventricle wall stress parameters. Clinical Kardiologie, 13:218-220, 1990.

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Nakatani, Y., C. Banner, M. **von Herrath**, E. Schneider, H. Smith and E. Freese. Comparison of human brain and liver glutamate dehydrogenase cDNAs. Biochem. Biophys. Res. Comm. 149:405-410, 1987.

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von Herrath, M.G. and H. Holzer. Oxidative inactivation of yeast fructose-1, 6-bisphosphatase. Intracell. Protein Catabolism 180:329-338, 1985.

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Argyrios Theofilopoulos, Professor
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Roger Beachy, Professor
Head, Division of Plant Biology
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Speakers Invited to TSRI:

Dr. Rolf Zinkernagel
Dr. Polly Matzinger
Dr. Trevor Owens
Dr. Kevin Lafferty
Dr. C.J. Peters
Dr. Lucienne Chatenoud
Dr. Carl Djerassi
Dr. Jonathan Stoye
Dr. Marc Jenkins
Dr. Steve Miller
Dr. Burce Walker

5/1996 - Faculty Seminar, Max Planck Institute for Immunobiology, Freiburg, FRG
Host: Professor H. Peter

5/1996 - Group Lecture, Department of Pathology, University of Cambridge, UK
Host: Prof. Anne Cooke

12/1996 - Group Seminar, Department of Clinical Immunology, University of Freiburg,
Host: Prof. H. Peter

6/1999 - Immunology Seminar Series, Stanford University, Host: Garrison Fathman

10/1999- Southwestrn Medical Center, Grand Rounds, Department of Dermatology, Dallas, TX, USA

37. A method for treating an autoimmune disease in a human or rodent host by suppressing an autoimmune response associated with said disease, the method comprising administering by nose or mouth to said host an effective amount for suppressing said response of a composition comprising a bystander antigen, wherein said bystander antigen is not an antigen to which T cells which mediate the disease are sensitized and wherein said bystander antigen is not an insulin antigen.

38. The method of claim 37 w
herein said bystander antigen is specific to an organ or tissue afflicted by immune attack during said disease.

Cancel claim 39 without prejudice or disclaimer.

42. The method of claim 37 wherein said bystander is administered to said host in aerosol form.

43. The method of claim 37 wherein said bystander antigen is administered in a dry powder form.

44. The method of claim 37 wherein said bystander antigen is administered as a saline solution.

Cancel claim 45 without prejudice or disclaimer.

46. The method of claim 38 wherein said disease is Type I diabetes and said bystander antigen is glucagon, administered orally.

Cancel claim 47 without prejudice or disclaimer.

48. A pharmaceutical dosage form for treating an autoimmune disease in a human or rodent, the form consisting essentially of:

an effective amount for treating said disease of a bystander antigen; and a pharmaceutically acceptable carrier or diluent;

wherein said bystander antigen is not insulin nor an antigen to which T cells that mediate said disease are sensitized, and wherein said dosage form is contained in an inhaler or nebulizer.

49. The pharmaceutical dosage form of claim 48 wherein said bystander antigen is specific to an organ or tissue afflicted by immune attack during said disease.

52. The pharmaceutical dosage form of claim 49 wherein said dosage form is an aerosol form.

53. The pharmaceutical dosage form of claim 49 wherein said dosage form is a saline solution.

54. The pharmaceutical dosage form of claim 49 wherein said dosage form is a dry powder.

Cancel claim 55 without prejudice or disclaimer.

56. The pharmaceutical dosage form of claim 48 wherein said disease is selected from the group consisting of Type I diabetes and animal models therefor and said bystander antigen is glucagon.

57. A pharmaceutical dosage form for nasal administration for treating Type I diabetes in a human comprising an effective amount for treating said type I diabetes of glutamic acid decarboxylase and a pharmaceutically acceptable carrier or diluent in an inhaler or nebulizer.

Cancel claims 59, 61-62 and 64-65 without prejudice or disclaimer.

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Immunology

Suppressor T cells generated by oral tolerization to myelin basic protein suppress both *in vitro* and *in vivo* immune responses by the release of transforming growth factor β after antigen-specific triggering

(tolerance/suppressor T cells/experimental autoimmune encephalomyelitis)

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ABSTRACT **Oral administration of myelin basic protein (MBP) is an effective way of suppressing experimental autoimmune encephalomyelitis (EAE). We have previously shown that such suppression is mediated by $CD8^+$ T cells, which adoptively transfer protection and suppress immune responses *in vitro*. In the present study we have found that modulator cells from animals orally tolerized to MBP produce a suppressor factor upon stimulation with MBP *in vitro* that is specifically inhibited by anti-transforming growth factor β (TGF-β) neutralizing antibodies. No effect was observed with antibodies to γ interferon, tumor necrosis factor α/β, or indomethacin. In addition, the active form of the type 1 isoform of TGF-β1 ($TGF-\beta_1$) can be directly demonstrated in the supernatants of cells from animals orally tolerized to MBP or ovalbumin after antigen stimulation *in vitro*. Antiserum specific for $TGF-\beta_1$ administered *in vivo* abrogated the protective effect of oral tolerization to MBP in EAE. Furthermore, injection of anti-$TGF-\beta_1$ serum to nontolerized EAE animals resulted in an increase in severity and duration of disease. These results suggest that immunomodulation of EAE induced by oral tolerization to MBP and natural recovery mechanisms use a common immunoregulatory pathway that is dependent on $TGF-\beta_1$. Implications of such an association are of therapeutic relevance to human autoimmune diseases and may help to explain one of the mechanisms involved in the mediation of active suppression by T cells.**

Immunological tolerance is the acquisition of unresponsiveness to self antigens and as such is essential for the preservation of the integrity of the host. A variety of mechanisms underly self-tolerance, including clonal deletion, clonal anergy, and active suppression (1), and its breakdown results in autoimmune diseases. The role and mechanism of action of active suppression in regulating the immune response are not well understood. One of the classic methods of inducing tolerance is via the oral administration of antigens, first described by Wells in 1911 (2) and subsequently by Chase in 1946 (3). We and others have been studying oral tolerance as a mechanism to suppress autoimmune processes in a number of experimental models (4–11, 49) and have found that oral administration of myelin basic protein (MBP) suppresses experimental autoimmune encephalomyelitis (EAE), a central nervous system autoimmune disease mediated by $CD4^+$ MBP-reactive cells (4–8). We have also found that this effect is mediated by active suppression. Specifically, $CD8^+$ T cells from animals orally tolerized to MBP suppress *in vitro* proliferative responses and adoptively transfer disease protection (5).

In the present investigation, we have found that T cells generated by oral tolerance mediate suppression both *in vitro* and *in vivo* via the release of the cytokine transforming growth factor-β (TGF-β). Our findings not only have relevance to orally induced tolerance but may help to explain one of the mechanisms by which active suppression mediated by T cells occurs.

MATERIALS AND METHODS

Animals. Female Lewis rats 6–8 weeks of age were obtained from Harlan–Sprague–Dawley. Animals were housed in Harvard Medical School animal care facilities and maintained on standard laboratory chow and water ad libitum.

Antigens. Guinea pig MBP was purified from brain tissue by the modified method of Deibler *et al.* (12). Protein content and purity were checked by gel electrophoresis and amino acid analysis.

Reagents. Commercial reagents used were as follows: mouse anti-rat γ interferon (INF-γ) neutralizing monoclonal antibody (mAb) (Amgen Biologicals); monoclonal hamster anti-murine TNF-α/β antibody (Genzyme); polyclonal rabbit neutralizing antibody against types 1 and 2 isoforms of TGF (antiTGF-β_{1+2}) (R & D Systems, Minneapolis), and indomethacin (Sigma). Turkey antiserum specific for $TGF-\beta_1$ was prepared as described (13).

Induction of Oral Tolerance. Rats were fed 1 mg of MBP dissolved in 1 ml of phosphate-buffered saline (PBS) or PBS alone by gastric intubation with a 18-gauge stainless steel animal feeding needle (Thomas Scientific). Animals were fed five times at intervals of 2–3 days with the last feeding 2 days before immunization.

***In Vitro* Suppression of Proliferative Responses by Supernatants.** Spleen cells were removed 7–14 days after the last feeding, and a single-cell suspension was prepared by pressing the spleens through a stainless steel mesh. Oral tolerance to MBP in the Lewis rat persists for ≈2 months after the last feeding (4). Thus, the 7- to 14-day period after feeding represents a relatively narrow window of time after oral tolerance is induced for performing the experiments. For preparation of supernatants, spleen cells at a concentration of 5×10^6 cells per ml were stimulated *in vitro* with MBP (50 μg/ml) in 10 ml of proliferation medium as described (20). Supernatants were harvested at 24 hr, and 100 μl was added

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Abbreviations: TGF, transforming growth factor; $TGF-\beta_1$ and $-\beta_2$, types 1 and 2 isoforms of TGF; MBP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; DTH, delayed-type hypersensitivity; INF-γ, γ interferon; mAb, monoclonal antibody; CFA, complete Freund's adjuvant.

to 2.5×10^4 MBP-specific T cells that had been raised and maintained as described (14) and cultured with 5×10^5 irradiated (2500 rad; 1 rad = 0.01 Gy) thymocytes in 100 μl of proliferation media. MBP (50 μg/ml) was added to the culture in a volume of 20 μl. Experiments were performed in triplicate in round-bottom 96-well plates (Costar). Cells were cultured for 72 hr at 37°C and harvested as described (20).

Purification of T-Cell Subsets. Depletion of lymphocyte subsets was performed by negative selection using magnetic beads according to a modified method of Cruikshank *et al.* (15). Spleen cells were incubated with a 1:10 dilution of mouse anti-rat CD8, CD4, or B-cell mAbs (clones OX/8, W3/25, or OX/33 respectively, Serotec Bioproducts) for 30 min on ice, washed twice, and then added to prewashed magnetic particles, with an average diameter of 4.5 μm (M-450) with goat anti-mouse IgG covalently attached (Dynal, Fort Lee, NJ). The cell–mAb–bead complexes were separated from unlabeled cells in a strong magnetic field with a magnetic-particle concentrator (Dynal MPC-1) for 2 min. The supernatant was removed, and the procedure was repeated twice to obtain the nonadherent fraction. The cells in the T cell- and B cell-depleted populations were >95% $CD4^+$ $CD8^-$, $CD4^-$ $CD8^+$, or $CD4^+$ $CD8^+$ $OX/33^-$ (B cell-depleted) as demonstrated by indirect flow cytometry. Whole spleen populations (5×10^6 cells) from MBP-fed or control animals were cultured in the presence of MBP (50 μg/ml) in 1 ml of serum-free proliferation media. Depleted populations were cultured at a concentration of 2.5×10^6 cells per ml. Supernatants were collected at 24 hr, and 100 μl was added to responder cells as described above.

Treatment of Supernatants with Anti-Cytokine Antibodies. Spleen cells (5×10^6 cells per ml in proliferation media) from MBP-fed and control animals were incubated in the presence of MBP (50 μg/ml) and neutralizing antibodies against INF-γ, TGF-β, TNF-α/β, or with indomethacin for 72 hr. Antibodies were tested in a range of concentrations (1:250, 1:500, 1:1000), and indomethacin was tested at concentrations of 0.5–1 μg/ml. At 24 hr, supernatants were collected, and free antibody or antibody–cytokine complexes were removed by using magnetizable polymer beads (Dynabeads; Dynal). Beads coupled with anti-immunoglobulin antibodies were incubated at a concentration of 4×10^7 beads per ml for 30 min (done twice for each sample) and were removed by a modified method of Liabakk *et al.* (16) using a Dynal Magnetic Particle Concentrator (MPC-1).

Measurement of TGF-β Activity in Serum-Free Culture Supernatants. Serum-free culture supernatants were collected as described (17, 18). Briefly, modulator cells were first cultured for 8 hr with MBP (50 μg/ml) in proliferation medium. Thereafter, cells were washed three times, resuspended in serum-free medium for the remainder of the 72-hr culture, collected, and then frozen until assayed. Determination of TGF-β content and isoform type in supernatants was performed by using a mink lung epithelial cell line (CCL-64; American Type Culture Collection) as described by Danielpour *et al.* (13) and was confirmed by a sandwich ELISA assay as described (19). The percent active TGF-β was assayed without prior acid activation of the samples.

Immunization of Animals. Lewis rats were immunized by injection in the left foot pad with 25 μg of MBP in 50 μl of PBS emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 4 mg of mycobacterium tuberculosis (Difco) per ml.

***In Vivo* Administration of Anti-TGF-β Antiserum and Control Sera.** Turkey antiserum specific for TGF-β_1 was used for *in vivo* experiments and had previously been prepared and characterized (13). Serum was heat-inactivated at 56°C for 30 min before injection. Animals (five per group) were injected i.p. with anti-TGF-β antiserum or control turkey serum at various concentrations (12.5, 25, or 50 μl diluted in PBS to a final volume of 100 μl), five times at days −2, 0, +2, +4, and +6 in relationship to MBP/CFA immunization. One microliter of the antiserum blocked the binding activity of ^{125}I-labeled TGF-β_1 at 4 ng/ml to A549 cells (13). *In vivo* treatment was given both to orally tolerized animals and to animals immunized for EAE without oral tolerization.

Clinical Evaluation. Animals were evaluated in a blinded fashion every day for evidence of EAE. Clinical severity of EAE was scored as follows: 0, no disease; 1, limp tail; 2, hind limb paralysis; 3, hind limb paraplegia and incontinence; 4, tetraplegia; and 5, death.

Delayed-Type Hypersensitivity (DTH) Testing. DTH was tested by injecting 25 μg of MBP in PBS subcutaneously in the ear. Thickness was measured by a blinded observer before and 48 hr after challenge with micrometer calipers (Mitutoyo, Utsunomia, Japan).

Statistical Analysis. Clinical scales were analyzed with a two-tailed Wilcoxon rank sum test for score samples, χ^2 analysis was used in comparing the incidence of disease between groups, and comparison of means was performed by using the Student *t* test.

RESULTS

***In Vitro* Suppression Is Mediated by Culture Supernatants from $CD8^+$ T Cells from MBP-Fed Animals.** In previous studies we have shown that modulator $CD8^+$ splenic T cells from MBP-fed animals suppress *in vitro* proliferation of an MBP-specific T-cell line (5). Furthermore, additional studies have demonstrated that such suppression occurs when modulator and responder cells are separated by a semipermeable membrane, suggesting the presence of a soluble factor as a mediator of the suppression (20). The generation of the soluble suppressor factor requires triggering of cells from orally tolerized animals with the oral tolerogen. Experiments were thus performed to determine whether supernatants collected from splenocytes depleted of T-cell subsets or B cells from rats orally tolerized to MBP and stimulated *in vitro*

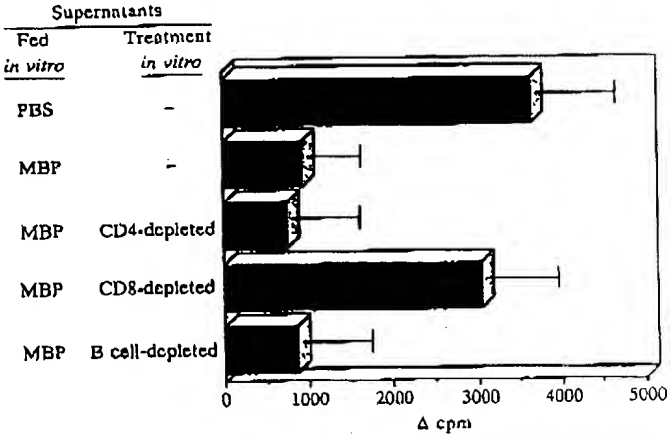

FIG. 1. *In vitro* suppression of proliferative response mediated by supernatants of lymphocyte subsets from orally tolerized animals. Whole spleen cells or depleted cell populations were stimulated *in vitro* with MBP (50 μg/ml). Spleen cells were depleted of B lymphocytes or $CD4^+$ or $CD8^+$ T lymphocytes by magnetized beads. One-hundred microliters of 24-hr culture supernatants of these cells was added to 2.5×10^4 MBP-specific T cells, cultured with 5×10^5 irradiated (2500 rad) thymocytes in proliferation medium containing MBP (50 μg/ml). Experiments were performed in triplicate in round-bottom 96-well plates. The proliferative response of the MBP-treated line in the presence of supernatants from MBP-stimulated spleen cells of nontolerized animals was 3654 ± 1651. Background counts of the MBP treated line in the absence of MBP were between 200 and 300 cpm.

with MBP could suppress an MBP line. A reduction in the proliferation of the MBP line occurred with the addition of supernatants from B cell-depleted or CD4-depleted splenocytes from animals fed MBP and stimulated *in vitro* with MBP (Fig. 1). No suppression occurred with supernatants from cells of PBS-fed animals or CD8-depleted splenocytes from MBP-fed animals.

Inhibition of *in Vitro* Suppression by Anti-TGF-β Antibodies. To determine whether a known cytokine was responsible for mediating the suppression, neutralizing antibodies to cytokines postulated to have suppressor activity were added to the supernatants in an attempt to abrogate the suppression. Rabbit anti-TGF-β antibody abrogated the suppression mediated by the supernatants in a dose-dependent fashion (Fig. 2). No effect on suppression was seen with neutralizing antibodies to INF-γ or TNF-α/β or when indomethacin, a prostaglandin blocker, was added. No suppression occurred when anti-TGF-β antibodies were added directly to the MBP-specific responder T-cell line (data not shown).

Demonstration of TGF-β in Culture Supernatants. To directly demonstrate the presence of TGF-β in supernatants of spleen cells from animals fed MBP and stimulated *in vitro* with MBP, we collected supernatants under serum-free conditions and assayed directly for TGF-β. TGF-β was secreted by spleen cells from MBP-fed animals stimulated *in vitro* in the presence but not in the absence of MBP (Fig. 3). Furthermore, TGF-β was also secreted when splenocytes from ovalbumin-fed animals are stimulated *in vitro* with ovalbumin. By using a specific sandwich ELISA assay with blocking antibodies specific for either TGF-β_1 or TGF-β_2 (13), it was further demonstrated that the TGF-β was of the TGF-β_1 isotype. In addition, the TGF-β secreted was in the active rather than latent form. The amount of TGF-β in the group fed and stimulated *in vitro* with MBP was 6.8 ± 1.7 ng/ml, with 68 ± 9% in the active form. In the ovalbumin-fed group, the amount of TGF-β was 6.1 ± 1.0 ng/ml, with 65 ± 9% in the active form. No active TGF-β was observed in supernatants from spleen cells of animals fed MBP and stimulated with concanavalin A, although small quantities (2.1 ± 0.45 ng/ml) of latent TGF-β were observed.

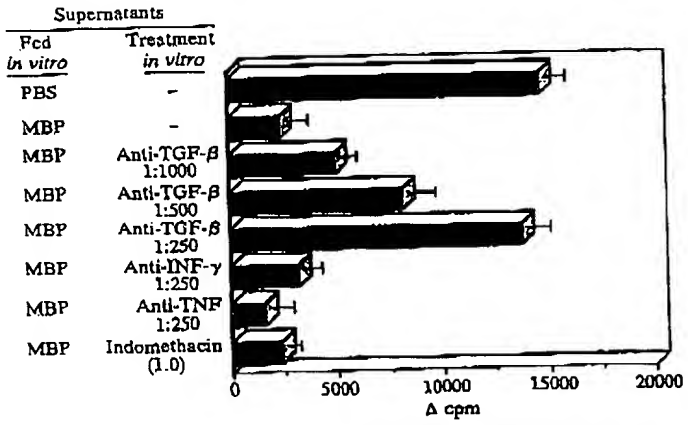

FIG. 2. Inhibition of *in vitro* suppression by anti-TGF-β antibody. Spleen cells from MBP-fed and control animals were incubated in the presence of MBP (50 μg/ml) and neutralizing antibodies against INF-γ, TGF-β, TNF-α/β, or with indomethacin for 72 hr. Free antibody or antibody–cytokine complexes from 24-hr supernatants of these cells were removed by using magnetizable polymer beads as described, and the suppressive effects of the treated supernatants were tested on an MBP-specific T-cell line. The proliferative response of the MBP line in the presence of supernatants from MBP-stimulated spleen cells of nontolerized animals was 18,995 ± 2395. Background counts of the MBP line in the absence of MBP were between 1000 and 2000 cpm.

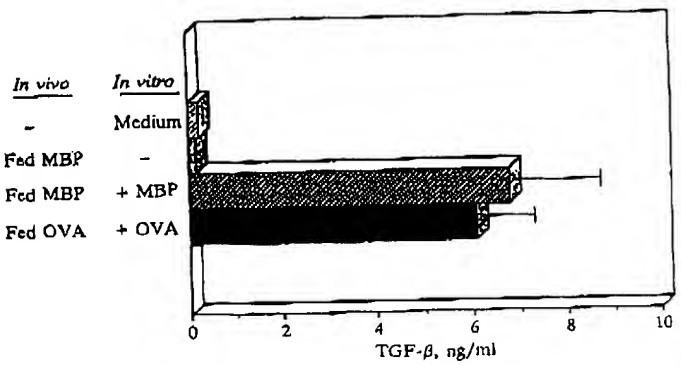

FIG. 3. TGF-β activity in serum-free culture supernatants. Spleen cells from MBP- or ovalbumin (OVA)-fed animals were incubated in the presence or absence of the homologous antigen (50 μg/ml). TGF-β content in the serum-free supernatants was assayed by using a CCL-64 assay as described.

Abrogation of Oral Tolerance by *in Vivo* Administration of Anti-TGF-β Antiserum. To determine whether TGF-β_1 also played a role in suppression of EAE by oral tolerization to MBP, we administered turkey anti-TGF-β_1 antiserum *in vivo*. Paralytic EAE developed in control animals with a maximal disease severity between 3.2 and 3.5 on day 13 regardless of whether animals were injected with PBS or control turkey serum (Fig. 4*A*). Oral tolerization with MBP markedly reduced the severity and duration of EAE (Fig. 4*C*) in animals injected with PBS or control turkey serum. Maximal disease severity in animals treated five times with 50 μl of control serum was 3.2 ± 0.2 and in orally tolerized animals treated five times with 50 μl of control serum was 1.0 ± 0.2 ($P < 0.05$). *In vivo* treatment with anti-TGF-β_1 antiserum abro-

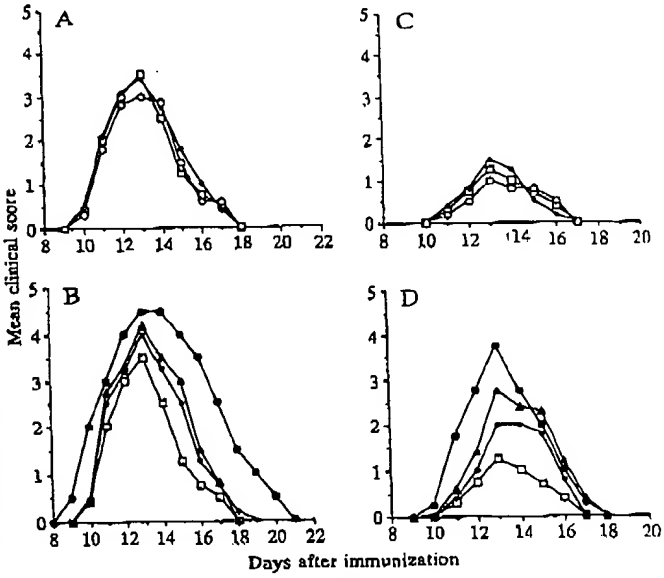

FIG. 4. The effect of anti-TGF-β and control sera on EAE. Animals received i.p. injections of turkey anti-TGF-β_1 antiserum or control preimmune turkey serum at various concentrations on days −2, 0, +2, +4, and +6 relative to the day of MBP/CFA challenge. Treatment was given both to orally tolerized animals (*C* and *D*) and to animals undergoing EAE without oral tolerance (*A* and *B*) (five animals per group). Maximal disease severity in orally tolerized animals treated five times with 50 μl of anti-TGF-β_1 antiserum was 3.7 ± 0.2 vs. 1.0 ± 0.2 in animals receiving control serum ($P < 0.05$) (*D* vs. *C*) and 4.5 ± 0.2 vs. 3.2 ± 0.2 in nontolerized animals ($P < 0.05$) (*B* vs. *A*). Treatments: □, PBS; ◇, control serum (12.5); ○, control serum (50); ◆, anti-TGF-β antiserum (12.5); ▲, anti-TGF-β antiserum (25); ●, anti-TGF-β antiserum (50).

gated protection induced by oral administration of MBP in a dose-dependent fashion; maximal disease severity in orally tolerized animals treated five times with 50 μl of anti-TGF-β_1 antiserum was 3.7 ± 0.2 vs. 1.0 ± 0.2 ($P < 0.05$; Fig. 4 *D* vs. *C*). Of note is that there was a dose-dependent enhancement of disease in animals treated with anti-TGF-β_1 antiserum that were not orally tolerized to MBP (Fig. 4*B*). Disease onset was earlier, recovery was delayed, and disease severity was greater (4.5 ± 0.2 vs. 3.2 ± 0.2, Fig. 4 *B* vs. *A*; $P < 0.05$).

DTH Responses Associated with *in Vivo* Anti-TGF-β Treatment. We have shown previously that DTH responses correlate with the clinical course of EAE and serve as a measure of *in vivo* cellular immunity to MBP (6, 7). In this study prominent DTH responses developed in animals undergoing EAE and DTH was suppressed by oral administration of MBP (Fig. 5). The suppressed DTH responses were abrogated by *in vivo* anti-TGF-β_1 treatment in a dose-dependent fashion (1.5 ± 0.5 vs. 0.5 ± 0.3; $P < 0.001$, in animals injected five times with 50 μl of anti-TGF-β vs. control serum). Furthermore, after the same *in vivo* treatment, there was enhancement of DTH responses to MBP in animals recovering from EAE that were not orally tolerized (2.1 ± 0.3 vs. 1.45 ± 0.3; $P < 0.01$, in animals injected five times with 50 μl of anti-TGF-β vs. control serum).

DISCUSSION

In the present study we found that T cells that mediate suppression of EAE after oral tolerization to MBP do so both *in vitro* and *in vivo* via the release of active TGF-β_1. *In vitro*, the release of TGF-β is dependent on antigen-specific triggering by the oral tolerogen whether it is MBP or ovalbumin. Among the mechanisms described for the maintenance of self-tolerance, active suppression mediated by T cells is probably the least well understood (21–23). Because oral tolerization represents a physiologic pathway by which the immune system is stimulated to generate suppression and because investigators have described active suppression mediated by T cells after oral tolerization (24, 25), an understanding of the mechanism by which suppressor cells act after stimulation of the gut-associated lymphoid tissue may provide insight into the mechanism of active suppression.

A number of cytokines exert suppressive activity on different aspects of the immune response such as cell growth, differentiation, and effector functions as well as the release of other cytokines. These include interferons, prostaglandins, tumor necrosis factor, and interleukin 10 (26). Our results show that $CD8^+$ suppressor T cells generated by oral tolerization, which are triggered in an antigen-specific fashion, act both *in vitro* and *in vivo* by the release of active TGF-β_1. In this regard they are analogous to the human $CD8^+$ suppressor cells associated with lepromatous leprosy, which act via an as-yet-unidentified antigen nonspecific factor after being triggered by a specific antigen (27). We recently have found that peripheral blood lymphocytes from humans orally tolerized to keyhole limpet hemocyaninin secrete a TGF-β-related suppressor factor after antigen-specific triggering (51). Because suppression generated by oral tolerance to autoantigens is antigen- and disease-specific, the secretion and action of TGF-β must occur in the local microenvironment of lymphoid tissue where the immune response is generated, along migratory pathways of the effector cells, and/or at the inflamed site in the target organ where the autoantigen is present. We recently have found specific elevation of TGF-β in the brains of EAE animals orally tolerized to MBP as compared to nontolerized animals (S. J. Khoury, W. W. Hancock, and H.L.W., unpublished observations). The ability of T cells to secrete TGF-β_1 in an active form may be important in this regard since active TGF-β has a short half-life and a small volume distribution, but latent TGF-β has an extended half-life and a larger volume of distribution (28).

We have also observed that injection of anti-TGF-β_1 serum to nontolerized animals immunized with MBP/CFA to induce EAE resulted in an increase in severity and duration of EAE. Other investigators have reported the presence of postrecovery $CD4^+$ suppressor cells in the Lewis rat EAE model that can adoptively transfer protection and suppress *in vitro* proliferative responses of an MBP line (29). *In vitro*, these cells have been shown to release TGF-β and to suppress interleukin-2 and IFN-γ production by encephalitogenic cells (29), although an *in vivo* effect of TGF-β by such cells has not been shown. Our *in vivo* results show a role for TGF-β in recovery from EAE. Thus, natural recovery in EAE and the induction of oral tolerance to MBP, though two distinct physiological processes, appear to involve a common immunoregulatory pathway in which TGF-β serves as an effector cytokine. The role of TGF-β-secreting $CD8^+$ T cells as opposed to $CD4^+$ T cells in natural recovery remains to be defined. TGF-β itself, when administered systematically in doses of 1–5 μg per treatment, has been shown to suppress

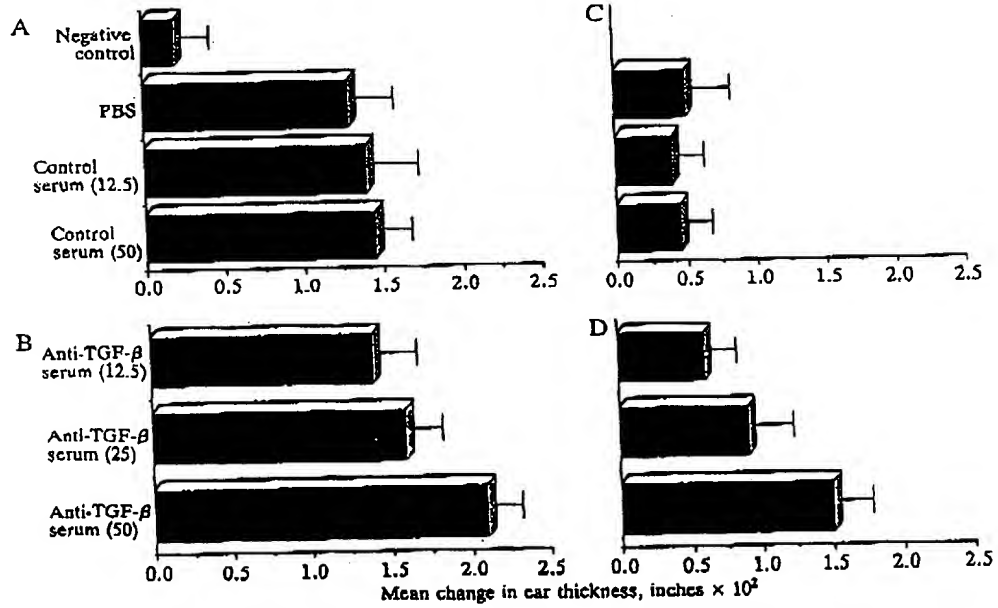

FIG. 5. DTH responses associated with *in vivo* anti-TGF-β treatment. DTH was tested in the groups described in Fig. 4 by injecting 25 μg of MBP in PBS subcutaneously in the ear. Thickness in inches (1 in. = 2.54 cm) was measured before and 48 hr after challenge. Change in ear thickness before and after challenge was recorded for each animal, and the results are expressed as the mean for each experimental group ± SEM. [$P < 0.001$, animals treated five times with 50 μl of anti-TGF-β_1 antiserum vs. control serum (*D* vs. *C*); $P < 0.01$, animals treated five times with 50 μl of anti-TGF-β_1 antiserum vs. control serum (*B* vs. *A*).]

animal models of autoimmunity both in the rat and mouse, including EAE (30, 31) and autoimmune arthritis (32, 33).

The mechanism by which TGF-β suppresses immune responses *in vitro* and down-regulates EAE and other autoimmune diseases *in vivo* is unknown. Recent studies have demonstrated multiple, sometimes contradictory, immunomodulatory effects of the TGF-β isoforms on various target cells and tissues (34, 35). Although initially identified as a growth factor, the immunoregulatory properties of TGF-β include inhibition of proliferation of B and T cells, (16, 36) affecting $CD4^+$ cells more than $CD8^+$ cells (37) in both rodent and human cells. TGF-β antagonizes inflammatory effector cytokines such as TNF-α and INF-γ (38, 39), blocks CTL activity (40, 50), and inhibits induction of receptors of interleukins 1 and 2 (41), rendering cells unresponsive to these cytokines. TGF-β inhibits *in vivo* T-cell and neutrophil adhesion to endothelial cells, which limits the migration and recruitment of inflammatory cells into the target organ (42, 43), downregulates class II expression on macrophages and astrocytes, and inhibits macrophage activation (40, 44).

TGF-β has been demonstrated to be secreted by a variety of cells including macrophages, natural killer cells, LAK cells, B cells, and both $CD4^+$ and $CD8^+$ T cells (45–47). The characteristics of the $CD8^+$ suppressor cells generated after oral tolerization are yet to be defined. Concanavalin A stimulation of spleen cells from animals tolerized with MBP, as opposed to MBP stimulation, did not lead to significant suppressor effects nor production of active TGF-β. This may be due to induction of proliferation rather than production of TGF-β after concanavalin A stimulation (48).

The results presented herein provide evidence for an immunoregulatory role played by endogenous TGF-β_1 in the spontaneously occurring recovery from EAE and in the suppression of EAE induced by oral tolerization to MBP. In view of its evolutionary high conserved features, it is likely that the immunosuppressive effects of TGF-β in experimental animals are similar to its effects in humans. Thus, our findings may point to the therapeutic potential of oral tolerization to MBP or other autoantigens as a source of endogenous TGF-β for the control of autoimmune diseases.

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2/4/92

CURRICULUM VITAE

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DATE OF BIRTH: September 17, 1947

PLACE OF BIRTH: Brooklyn, New York

EDUCATION

1969	B.A., Columbia University, New York, NY
1974	Ph.D., Duke University, Durham, NC (Physiology-Pharmacology)
1975	M.D., Duke University School of Medicine

INTERNSHIP AND RESIDENCIES AND FELLOWSHIPS

1975	Intern, Duke University Medical Center
1976	Junior Assistant Resident in Medicine, Duke University Medical Center
1/1977-6/77	Fellow in Endocrinology, Duke University Medical Center
1977-79	Research Associate, National Heart, Lung and Blood Institute, Laboratory of Biochemical Genetics (M. Nirenberg, A. Fauci), NIH, Bethesda, MD
1977-79	Fellow, Combined Endocrinology Program Clinic, NIH

LICENSURE AND CERTIFICATION

1976	National Board of Medical Examiners, No. 126840
1978	American Board of Internal Medicine, No. 63999
1979	Diplomate, Endocrinology and Metabolism, No. 63999
1982	Massachusetts License, Registration No. 49856

ACADEMIC APPOINTMENTS

1979-82	Assistant Professor of Medicine & Physiology, Duke University
1982-	Associate Professor of Medicine, Harvard Medical School, Boston, MA

HOSPITAL APPOINTMENTS

1979-82	Assistant Professor of Medicine, Duke University
1982-	Associate Physician, Brigham and Women's Hospital, Harvard Medical School
1983-	Associate on Staff, New England Deaconess Hospital, Boston, MA

OTHER PROFESSIONAL POSITIONS

1982-	Senior Investigator, Elliot P. Joslin Research Laboratories, Joslin Diabetes Center, Boston, MA
1982-	Head, Immunology Section, Research Division, Joslin Diabetes Center
1987-1988	Chairman, American Diabetes Association Research Committee
1987-1991	Member, Immunologic Sciences Study Section, NIH
1990-	Chairman elect Immunology of Diabetes Workshop (IDW) Committee
1990-	Consultant Calbio, Immunologic, Xoma, Autoimmune
1990-1991	Acting Chief, Diabetes Division, Brigham and Women's Hospital, Boston, MA
1991-	Advisory Board Duke University Arthritis Center

AWARDS AND HONORS

1965	Pulitzer Scholarship, Columbia University, NY
1981-84	Career Development Awardee, Juvenile Diabetes Foundation
1984	Weitzman Memorial Award, American Endocrine Society
1986	Outstanding Scientific Achievement Award of the American Diabetes Association and the Lilly Lecture
1987	JDF Mary Jane Kugel Award
1987	Peter J. Laurie Memorial Lecture
1988	JDF Don Silver Excellence in Research Award
1989	Louise Buerki Visiting Professor, Henry Ford Hospital
1989	Fourteenth Annual Arnold Lazarow Memorial Lecture, University of Minnesota
1990	Otto Brandman Award, ADA, New Jersey Affiliate

MEMBERSHIPS, OFFICES AND COMMITTEE ASSIGNMENTS

1979	American College of Physicians
	American Federation of Clinical Research
	The Endocrine Society
1982	American Society of Clinical Investigation
	American Diabetes Association
1983-88	American Diabetes Association Research Committee
1983-85	Editorial Board Diabetes
1984-87	Juvenile Diabetes Foundation, Medical Science Review Committee
1985	Second Vice Chairman, American Diabetes Association Research Committee
1987	Chairman, American Diabetes Association, Research Committee
1985-88	Treasurer IDW (Immunology of Diabetes Workshop)

MEMBERSHIPS, OFFICES AND COMMITTEE ASSIGNMENTS (Contd.)

1987-89	American Diabetes Association Research Policy Committee
1987	Chairman, ADA Research Symposium, VIII International Conference on the Immunology of Diabetes, Woods Hole, MA
1986-	Scientific Board, Barbara Davis Childhood Diabetes Center, Denver, Colorado
1987-91	Immunologic Sciences Study Section, National Institutes of Health
1986-	Board of Governors, American Diabetes Association, Massachusetts Affiliate
1987-	Editorial Board, Journal of Autoimmunity
1986-89	Board of Trustees, Joslin Diabetes Center
1986-	Associate Director, and Director of Cores, Diabetes Endocrine Research Center, Joslin Diabetes Center
1987-	Metabio Scientific Advisory Board
1988-	Editorial Board, Journal of Clinical Immunology
1988-91	Chairman-Elect, Immunology of Diabetes Workshops Committee
1988-91	Chairman Abstract Subcommittee of the 14th International Diabetes Federation Congress, Washington DC
1989-90	National Diabetes Advisory Council, Diabetes Treatment Centers of America Foundation
1989-	Consultant, CW Group
1989-	Consultant, Xoma Corporation
1989-1990	National Institutes of Allergy and Infectious Diseases Task Force on Immunology and Allergy, chapter co-chair
1990	Chairman Elect IDW Committee
1990	CRC Advisory Committee Brigham and Womens
1991-	Associate Editor, Diabetes
1991-95	National Institute of Health Reviewers Reserve.
1991	External Advisor, Duke Specialized Center of Research in Rheumatoid Arthritis.

MAJOR RESEARCH INTERESTS

Immunoendocrinology
Immunogenetics
Islet Cell Culture

TEACHING EXPERIENCE

1979-82	Lecturer for the Physiology and Endocrinology courses to medical students at Duke University
1979-82	Attending Physician, Medical Service, Duke University
1979-82	Coordinator, Endocrine Research Conference, Duke University Medical Center
1982-	Endocrinology/Diabetes Consultant, Brigham & Women's Hospital
1983-84	Coordinator, Joslin Research Seminar
1983-	Attending Physician, Medical Service, New England Deaconess Hospital
1985-	Attending Diabetes Treatment Unit, Joslin Diabetes Center
1989-	Invited Lecturer, Pharmacology Medical Student Course, MIT
1991	Max Miller Lecture, 2nd Annual Midwest Metabolism Club Meeting, The University of Chicago Medical Center

PRINCIPAL CLINICAL AND HOSPITAL SERVICE RESPONSIBILITIES

1983-85	Human Investigations Committee, New England Deaconess Hospital
1983-85	Human Investigations Committee, Joslin Diabetes Center
1984-85	Bylaws Committee, Joslin Diabetes Center
1985-88	Operations Committee, Joslin Diabetes Center
1989-	Institutional Review Board, Brigham and Womens Hospital
1989-	Institutional Review Board, Brigham and Womens Hospital
1992-	McLaughlin Lecture, The University of Texas Medical Branch at Galveston

ORIGINAL REPORTS

1. Delcher HK, Eisenbarth GS, Lebovitz HE. Fatty acid inhibition of sulfation factor stimulated 35S04 incorporation to embryonic chick cartilage. J Biol Chem 1973; 248:1901-1905.

2. Birch BM, Delcher HK, Rendall JL, Eisenbarth GS, Lebovitz HE. Evidence that endogenous cyclic AMP does not modulate serum sulfation factor action on embryonic chicken cartilage. Biochem Biophys Res Comm 1973; 52:1184-1189.

3. Eisenbarth GS, Beuttel SC, Lebovitz HE. Fatty acid inhibition of somatomedin (serum sulfation factor) stimulated protein and RNA synthesis in embryonic chicken cartilage. Biochem Biophys Acta 1973; 331:397-409.

4. Eisenbarth GS, Lebovitz HE. Prostaglandin inhibition of cartilage chrondromucoprotein synthesis: concept of intrinsic activity. Prostaglandins 1974; 7:11-20.

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11. Eisenbarth GS, Wilson P, Ward F, Lebovitz HE. HLA type and disease occurrence in familial poly-glandular failure. N Engl J Med 1978; 298:92-94.

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24. Eisenbarth GS, Oie H, Gazdar Z, Chick WL, Schultz JA, Scearce RM. Production of monoclonal antibodies reacting with rat islet cell membrane antigens. Diabetes 1981; 30:226-230.

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55. Srikanta S, Ganda OP, Gleason RE, Jackson RA, Soeldner JS, Eisenbarth GS. Pre-Type I diabetes: linear loss of beta cell response to intravenous glucose. Diabetes 1984; 33:717-720.

56. Buse JB, Ben-Nun A, Klein KA, Eisenbarth GS, Seidman JG, Jackson RA. Specific class II histocompatibility gene polymorphism in BB rats. Diabetes 1984; 33:700-703.

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ORIGINAL REPORTS (Contd.)

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62. Eisenbarth GS, Fleischnick E, Ganda OP, Srikanta S, Jackson RA, Brink SJ, Soeldner JS, Yunis E, Alper C. Progressive autoimmune beta cell insufficiency: occurrence in the absence of high risk HLA alleles DR3, DR4. Diabetes Care 1985; 8:477-480.

63. Nayak RC, Omar MAK, Rabizadeh A, Srikanta S, Eisenbarth GS. "Cytoplasmic" islet cell antibodies: evidence that the target antigen is a sialoglycoconjugate. Diabetes 1985; 34:617-619.

64. Buse JB, Rifai-Haddad R, Lees S, Taniguchi H, Chaplin D, Milford E, Seidman J, Eisenbarth GS, Jackson RA. Major histocompatibility complex restriction fragment length polymorphisms define three diabetogenic haplotypes in BB and BBN rats. J Exp Med 1985; 162:444-458.

65. Srikanta S, Ganda OP, Soeldner JS, Eisenbarth GS. First degree relatives of patients with Type I diabetes mellitus: islet cell antibodies and abnormal insulin secretion. N Engl J Med 1985; 313:461-464.

66. Eisenbarth GS, Srikanta S, Jackson RA, Rabinowe SL, Dolinar R, Haynes BF, Morris MA. ATGAM and prednisone immunotherapy of recent onset Type I diabetes mellitus. Diabetes Res 1985; 2:271-276.

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ORIGINAL REPORTS (Contd.)

71. Hattori M, Buse JB, Jackson RA, Glimcher L, Makino S, Moriwaki K, Dorff M, Minami M, Kuzuya H, Imura H, Seidman JG, Eisenbarth GS. The NOD mouse: recessive diabetogenic gene within the major histo-compatibility complex. Science 1986; 231:733-735.

72. Srikanta S, Ricker AT, McCulloch DK, Soeldner JS, Eisenbarth GS, Palmer JP. Autoimmunity to insulin, beta cell dysfunction and development of insulin dependent diabetes mellitus. Diabetes 1986; 35:139-142.

73. Rabinowe SL, George KL, Laughlin R, Soeldner JS, Eisenbarth GS. Congenital rubella: monoclonal antibody defined T cell abnormalities in young children. Am J Med 1986; 81:779-782.

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ORIGINAL REPORTS (Contd.)

81. Dib SA, Colman PG, Dotta F, Tautkus M, Rabizadeh A, Eisenbarth GS. Expression of "cytoplasmic" islet cell antigens by rat pancreas. Diabetes 1987; 36:982-985.

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100. Rabinowe SL, Rubin L, George KL, Adri MNS, Eisenbarth GS. Trisomy 21 (Down's syndrome): autoimmunity, aging and monoclonal antibody defined T cell abnormalities. J Autoimmunity 1989; 2:25-30.

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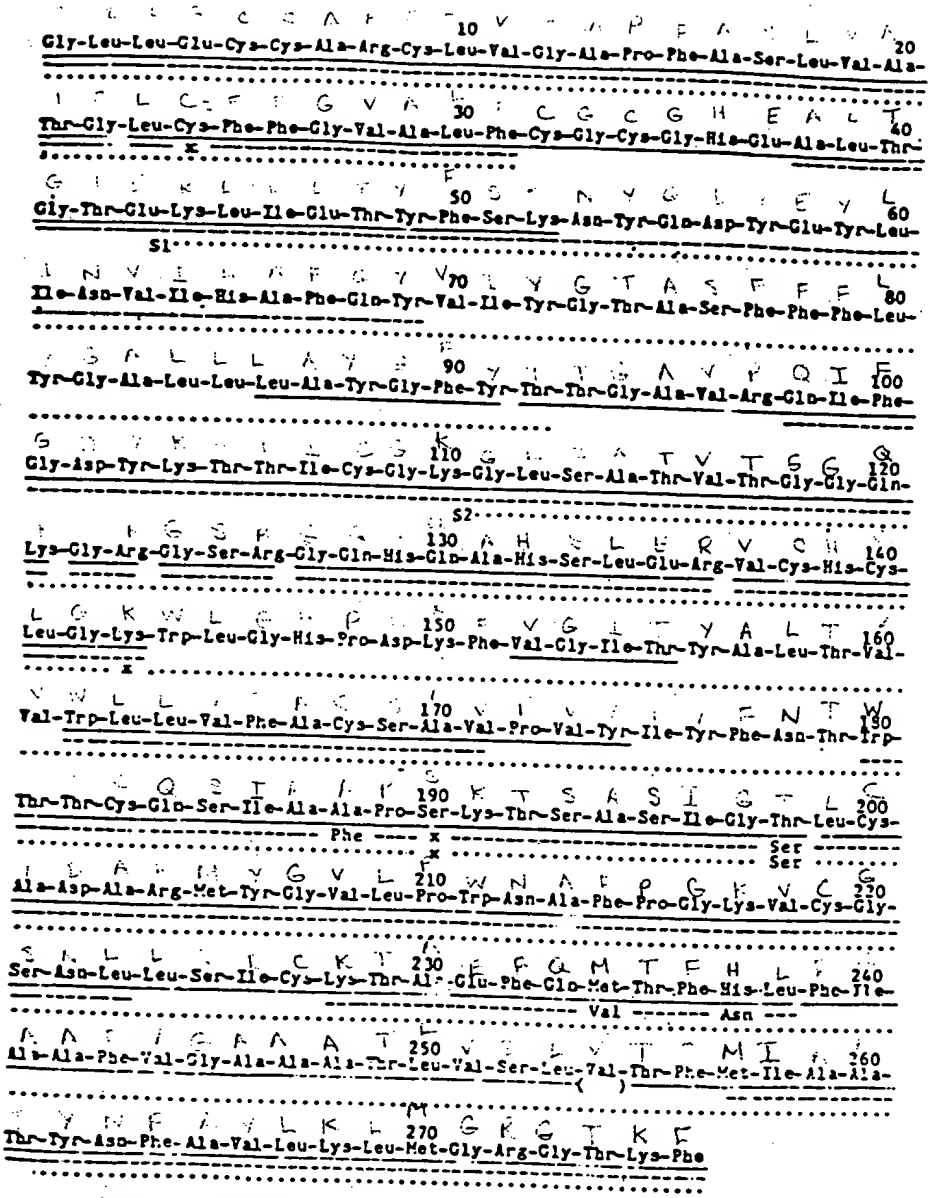
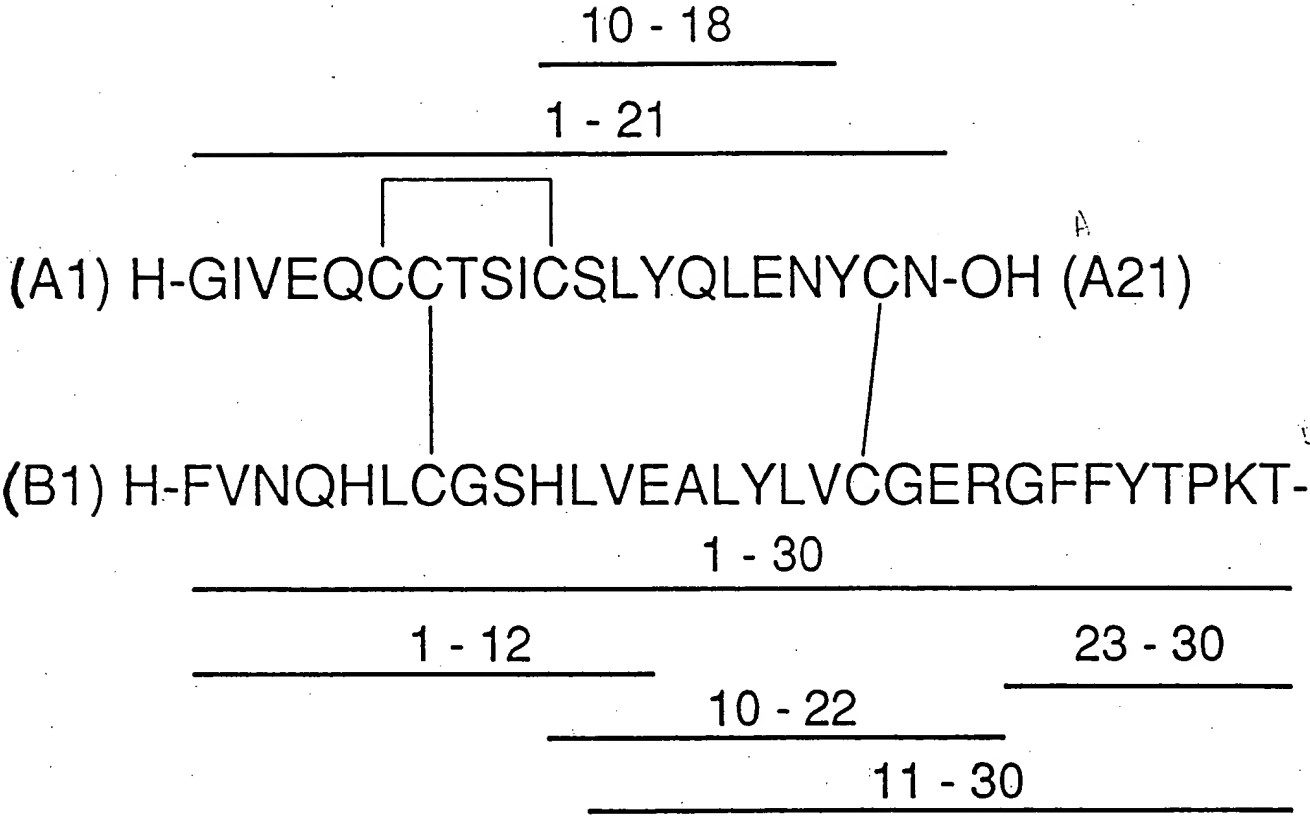
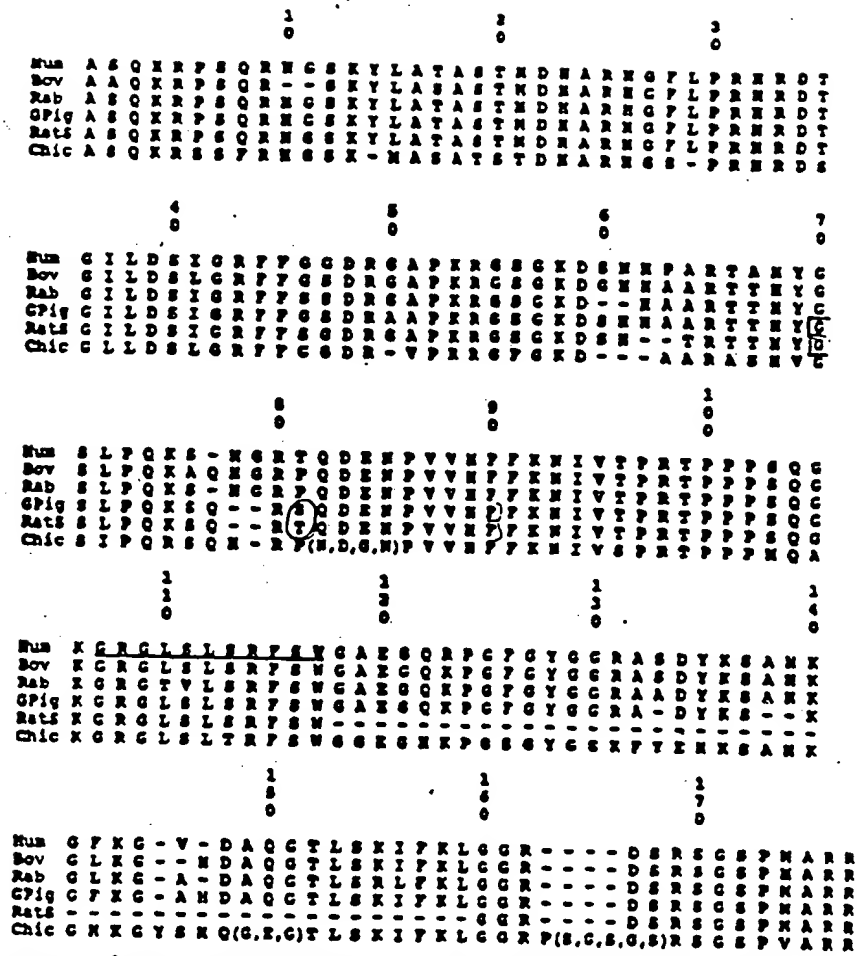
APPENDIX A



Sequence of bovine PLP.

INSULIN FRAGMENTS





Amino acid sequences of myelin basic proteins derived from human (Hum), bovine (Bov), rabbit (Rab), guinea pig (GPig), rat (RatS), and chicken (Chic) central nervous system tissue. The rat BP used was the 14 kDa BP (rat small, or RatS) which has a deletion of residues 118–159, coded for by the 6th exon of the BP gene. Both the human 18.5 and 17.2 kDa forms of BP were used. The latter has a deletion of residues 107–117 (underlined), coded for by the 5th exon of the BP gene. The sequences are arranged such that homologous residues from each species are arranged vertically, so that they can easily be compared with one another. This system accommodates the deletions and additions that are found among the species, and allows for a total of 177 potential sites among the different molecules. The sequences of residues in parentheses have not been established.

The comparative amino acid sequences:

Human α1(II)	Bovine α1(II)	Bovine α1(I)	
gly pro met	gly VAL met	gly pro met	
gly pro met	gly pro met	gly pro SER	105
gly pro arg	gly pro arg	gly pro arg	
gly pro pro	gly pro pro*	gly LEU pro*	
gly pro ala	gly pro ala	gly pro PRO*	
gly ala pro	gly ala pro*	gly ala pro*	
gly pro gln	gly pro gln	gly pro gln	
gly phe gln	gly phe gln	gly phe gln	
gly asn pro	gly asn pro	gly PRO pro	
gly glu pro	gly glu pro*	gly glu pro*	
gly glu pro	gly glu pro*	gly glu pro*	132
gly val ser	gly val ser	gly ALA ser	
gly pro met	gly pro met	gly pro met	
gly pro arg	gly pro arg	gly pro arg	
gly pro pro	gly pro pro*	gly pro pro*	
gly pro pro	gly pro pro*	gly pro pro*	
gly lys pro	gly lys pro*	gly lys ASN	
gly asp asp	gly asp asp	gly asp asp	
gly glu ala	gly glu ala	gly glu ala	
gly lys pro	gly lys pro*	gly lys pro*	
gly lys ala	gly lys SER	gly ARG PRO*	162
gly glu arg	gly glu arg	gly glu arg	
gly pro pro	gly pro pro*	gly pro pro*	
gly pro gln	gly pro gln	gly pro gln	
gly ala arg	gly ala arg	gly ala arg	
gly phe pro	gly phe pro*	gly LEU pro*	
gly thr pro	gly thr pro*	gly thr ALA	
gly leu pro	gly leu pro*	gly leu pro*	
gly val lys	gly val lys*-glc-gla	gly MET lys*-glc-gla	
gly his arg	gly his arg	gly his arg	
gly tyr pro	gly tyr pro*	gly PHE SER	192
gly leu asp	gly leu asp	gly leu asp	
gly ala lys	gly ala lys*-glc-gla	gly ala lys*-glc-gla	
gly glu ala	gly glu ala	gly ASP ala	
gly ala pro	gly ala pro*	gly PRO ALA	
gly val lys	gly val lys	gly PRO lys	
gly glu ser	gly glu ser	gly glu PRO*	
gly ser pro	gly ser pro*	gly ser pro*	
gly glu asn	gly glu asn	gly glu asn	
gly ser pro	gly ser pro*	gly ALA pro*	
gly pro met	gly pro met	gly GLN met	222
gly pro arg	gly pro arg	gly pro arg	
gly leu pro	gly leu pro*	gly leu pro*	
gly glu arg	gly glu arg	gly glu arg	
gly arg thr	gly arg thr	gly arg PRO*	

gly pro ala	gly pro ala	gly pro PRO*
gly ala ala	gly ala ala	gly SER ala
gly ala arg	gly ala arg	gly ala arg
gly asn asp	gly asn asp	gly ASP asp
gly gln pro	gly gln pro*	gly ALA VAL
gly pro ala	gly pro ala	gly ALA ala
gly pro pro	gly pro pro*	gly pro pro*
gly pro val	gly pro val	gly pro THR
gly pro ala	gly pro ala	gly pro ala
gly gly pro	gly gly pro*	gly PRO pro*
gly phe pro	gly phe pro*	gly phe pro*
gly ala pro	gly ala pro*	gly ala VAL
gly ala lys	gly ala lys*-glc-gla	gly ala lys*-glc-gla
gly glu ala	gly glu ala	gly glu GLY
gly pro thr	gly pro thr	gly pro thr
gly ala arg	gly ala arg	gly PRO arg
gly pro glu	gly pro glu	gly SER glu
gly ala gln	gly ala gln	gly PRO gln
gly pro arg	gly pro arg	gly VAL arg
gly glu pro	gly glu pro*	gly glu pro*
gly thr pro	gly thr pro*	gly PRO pro*
gly ser pro	gly ALA pro*	gly PRO ALA
gly pro ala	gly pro ala	gly ALA ala
gly ala ser	gly ala ALA	gly PRO ALA
gly asn pro	gly asn pro*	gly asn pro*
gly thr asp	gly ALA asp	gly ALA asp
gly ile pro	gly ile pro*	gly GLU pro*
gly ala lys	gly ala lys*	gly ala lys*
gly ser ala	gly ser ala	gly ALA ASN
gly ala pro	gly ala pro*	gly ala pro*
gly ile ala	gly ile ala	gly ile ala
gly ala pro	gly ala pro*	gly ala pro*
gly phe pro	gly phe pro*	gly phe pro*
gly pro arg	gly ALA arg	gly ALA arg
gly pro pro	gly pro pro*	gly pro SER
asp pro gln	GLY PRO THR	GLY PRO GLN
gly ala thr	gly ala SER	gly ala PRO
gly pro leu	gly pro leu	gly pro PRO*
gly pro lys	gly pro lys*	gly pro lys*
gly gln thr	gly gln thr	gly ASN SER
gly lys pro	gly lys pro	gly lys pro
gly ile ala	gly ile ala	gly ALA PRO*
gly phe lys	gly phe lys*	gly ASN lys*
gly glu gln	gly glu gln	gly ASP THR
gly pro lys	gly pro lys*	gly ALA lys*
gly glu pro	gly glu pro*	gly glu pro*
gly pro ala	gly pro ala	gly pro THR
gly pro gln	gly VAL gln	gly ILE gln
gly ala pro	gly ala pro*	gly PRO pro*

252

282

312

342

372

gly pro ala
gly glu glu
gly lys arg
gly ala arg
gly glu pro
gly gly val
gly pro ile
gly pro pro
gly glu arg
gly ala pro
gly asn arg
gly phe pro
gly gln asp
gly leu ala
gly pro lys
gly ala pro
gly glu arg
gly pro ser
gly leu ala
gly pro lys
gly ala asn
gly asp pro
gly arg pro
gly glu pro
gly leu pro
gly ala arg
gly leu thr
gly arg pro
gly asp ala
gly pro gln
gly lys val
gly pro ser
gly ala pro
gly glu asp
gly arg pro
gly pro pro
gly pro gln
gly ala arg
gly gln pro
gly val met
gly phe pro
gly pro lys
gly ala asn
gly glu pro
gly lys ala
gly glu lys
gly leu pro
gly ala pro
gly leu arg

gly pro ala
gly glu glu
gly lys arg
gly ala arg
gly glu pro*
gly gly ALA
gly pro ALA
gly pro pro*
gly glu arg
gly ala pro*
gly SER arg
gly phe pro*
gly gln asp
gly leu ala
gly pro lys*
gly PRO pro*
gly glu arg
gly SER PRO*
gly ALA VAL
gly pro lys*
gly SER PRO*
gly GLU ALA
gly arg pro*
gly glu ALA
gly leu pro*
gly ala LYS*
gly leu thr
gly arg pro*
gly asp ala
gly pro gln
gly lys val
gly pro ser
gly ala pro*
gly glu asp
gly arg pro*
gly pro pro*
gly pro gln
gly ala arg
gly gln pro*
gly val met
gly phe pro*
gly pro lys*
gly ala asn
gly glu pro*
gly lys ala
gly glu lys*
gly leu pro*
gly ala pro*

gly pro ala
gly glu glu
gly lys arg
gly ala arg
gly glu pro*
gly PRO THR
gly LEU PRO*
gly pro pro*
gly glu arg
gly GLY pro*
gly SER arg
gly phe pro*
gly ALA asp
gly VAL ala
gly pro lys*
gly PRO ALA
gly glu arg
gly ALA PRO*
gly PRO ALA
gly pro lys*
gly SER PRO*
gly GLU ALA
gly arg pro*
gly glu ALA
gly leu pro*
gly ala LYS*
gly leu thr
gly SER pro*
gly SER PRO*
gly pro ASP
gly lys THR
gly pro PRO*
gly PRO ALA
gly GLN ASN
gly arg pro*
gly pro pro*
gly pro PRO*
gly ala arg
gly gln ALA
gly val met
gly phe pro*
gly pro LYS
gly ala ALA
gly glu pro*
gly lys ala
gly glu ARG
gly VAL pro*
gly PRO pro*

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492
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gly leu pro
gly lys asp
gly glu thr
gly ala glu
gly pro pro
gly pro ala
gly pro ala
gly glu arg
gly glu gln
gly ala pro
gly pro ser
gly phe gln
gly leu pro
gly pro pro
gly pro pro
gly glu ala
gly lys pro
gly asp gln
gly val pro
gly glu ala
gly ala pro
gly leu val
gly pro arg
gly glu arg
gly phe pro
gly glu arg
gly ser pro
gly ala gln
gly leu gln
gly pro arg
gly leu pro
gly thr pro
gly thr asp
gly pro lys
gly ala ser
gly pro ala
gly pro pro
gly ala gln
gly pro pro
gly leu gln
gly met pro
gly glu arg
gly ala ala
gly ile ala
gly pro lys
gly asp arg
gly asp val
gly glu lys
gly pro glu

552

582

612

642

672

gly thr asp	gly thr asp	gly ASN asp
gly pro lys	gly pro lys*	gly ALA lys*
gly ala ser	gly ala ALA	gly ASP ALA
gly pro ala	gly pro ala	gly ALA PRO*
gly ile ala	gly ile ala	gly LEU PRO*
gly pro lys	gly pro lys*	gly pro LYS
gly asp arg	gly asp arg	gly asp arg
gly asp val	gly asp val	gly asp ALA
gly glu lys	gly glu lys	gly PRO lys
gly pro glu	gly pro glu	gly ALA ASP

gly pro pro
gly ser pro
gly pro ala
gly pro thr
gly lys gln
gly asp arg
gly glu ala
gly ala gln
gly pro met
gly pro ser
gly pro ala 1002
gly ala arg
gly ile gln
gly pro gln
gly pro arg
gly asp lys
gly glu ala
gly glu pro
gly glu arg
gly leu lys
gly his arg 1032
gly phe thr
gly leu gln
gly leu pro
gly pro pro
gly pro ser
gly asp gln
gly ala ser
gly pro ala
gly pro ser
gly pro arg 1062
gly pro pro
gly pro val
gly pro ser
gly lys asp
gly ala asn
gly ile pro
gly pro ile
gly pro pro
gly pro arg
gly arg ser 1092
gly glu thr
gly pro ala
gly pro pro
gly asn pro
gly pro pro
gly pro pro
gly pro pro
gly pro gly

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gly ala pro
gly lys asp
gly ala arg
gly leu thr
gly pro ile
gly pro pro
gly pro ala
gly ala asn
gly glu lys
gly glu val
gly pro pro
gly pro ala
gly ser ala
gly ala arg
gly ala pro
gly glu arg
gly glu thr
gly pro pro
gly pro ala
gly phe ala
gly pro pro
gly ala asp
gly gln pro
gly ala lys
gly glu gln
gly glu ala
gly gln lys
gly asp ala
gly ala pro
gly pro gln
gly pro ser
gly ala pro
gly pro gln
gly pro thr
gly val thr
gly pro lys
gly ala arg
gly ala gln
gly pro pro
gly ala thr
gly phe pro
gly ala ala
gly arg val
gly pro pro
gly ser asn
gly asn pro
gly pro pro
gly pro pro
gly pro ser

gly ala pro

gly ASP VAL
gly glu lys*
gly glu val
gly pro pro*

gly gln pro
gly ala lys*
gly GLY gln
gly glu ala
gly gln lys*
gly asp ala
gly ala pro*

gly ala pro

gly ALA PRO*
gly ASP LYS
gly glu ALA
gly pro SER

gly gln PRO*
gly ala LYS
gly GLU PRO*
gly ASP ala
gly ALA lys*
gly asp ala
gly ala pro*

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762

792

822

gly lys asp
gly pro lys
gly ala arg
gly asp ser
gly pro pro
gly arg ala
gly glu pro
gly leu gln
gly pro ala
gly pro pro 852
gly glu lys
gly glu pro
gly asp asp
gly pro ser
gly ala glu
gly pro pro
gly pro gln
gly leu ala
gly gln arg
gly ile val 882
gly leu pro
gly gln arg
gly glu arg
gly phe pro
gly leu pro
gly pro ser
gly glu pro
gly gln gln
gly ala pro
gly ala ser 912
gly asp arg
gly pro pro
gly pro val
gly pro pro
gly leu thr
gly pro ala
gly glu pro
gly arg glu
gly ser pro
gly ala asp 942
gly pro pro
gly arg asp
gly ala ala
gly val lys
gly asp arg
gly glu thr
gly ala val
gly ala pro
gly ala pro
972